FORM PTO-1390 (Modified) (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **0010-1075-0 PCT**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): **09/462472**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP98/03239	**17 JULY 1998**	**18 JULY 1997**

TITLE OF INVENTION
METHOD FOR PRODUCING PURINE NUCLEOSIDE BY FERMENTATION

APPLICANT(S) FOR DO/EO/US
Hiroshi MATSUI, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 18 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
    A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☐ Certificate of Mailing by Express Mail
19. ☒ Other items or information:

Request for Consideration of Documents Cited in International Search Report
Notice of Priority
Drawings (3 Sheets)
Sequence Listing (Pages 1- 9)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/462472	PCT/JP98/03239	0010-1075-0 PCT

20. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		CALCULATIONS PTO USE ONLY
☒ Search Report has been prepared by the EPO or JPO	$840.00	
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482)	$670.00	
☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	$760.00	
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO	$970.00	
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	$96.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =		$840.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	13 - 20 =	0	x $18.00	$0.00
Independent claims	1 - 3 =	0	x $78.00	$0.00
Multiple Dependent Claims **(check if applicable).**			☐	$0.00
TOTAL OF ABOVE CALCULATIONS			=	$840.00
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable).**			☐	$0.00
SUBTOTAL			=	$840.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).			+	$0.00
TOTAL NATIONAL FEE			=	$840.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**			☐	$0.00
TOTAL FEES ENCLOSED			=	$840.00
			Amount to be: refunded	$
			charged	$

☒ A check in the amount of **$840.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **15-0030** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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January 14, 2000
DATE

DOCKET NO. 0010-1075-0 PCT

09/462472
420 Rec'd PCT/PTO 14 JAN 2000

IN RE APPLICATION OF: Hiroshi MATSUI, et al.

SERIAL NO.: NEW U.S. PCT APPLICATION(based on PCT/JP98/03239)

FILED: HEREWITH

FOR: METHOD FOR PRODUCING PURINE NUCLEOSIDE BY FERMENTATION

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

Sir:

Transmitted herewith is an amendment in the above-identified application.

☒ No additional fee is required.

☐ Small entity status of this application under 37 C.F.R. §1.9 and §1.27 has been established by a verified statement previously submitted.

☐ Small entity status of this application under 37 C.F.R. §1.9 and §1.27 has been established by a verified statement submitted herewith.

☒ Additional documents filed herewith: English Translation of Specification/PCT Transmittal Letter
Declaration/Preliminary Amendment/International Search Report/Request for Consideration
Notice of Priority/Sequence Listing (Pages 1-9)/Check for $840.00

The fee has been calculated as shown below.

	(Col. 1)		(Col. 2)	(Col. 3)	SMALL ENTITY		OTHER THAN A SMALL ENTITY	
	CLAIMS REMAINING AFTER		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE	ADDITIONAL FEE	RATE	ADDITIONAL FEE
TOTAL	* 13	MINUS	** 20	= 0	X9 =	$	X18 =	$ .00
INDEP	* 1	MINUS	*** 3	= 0	X39 =	$	X78 =	$ .00
☐	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				+130=	$	+260=	$
					TOTAL	$	TOTAL	$ .00

__ A check in the amount of $_____ is attached.

XX Please charge any additional fees for the papers being filed herewith and for which no check is enclosed herewith, or credit any overpayment to deposit Account No. 15-0030. A duplicate copy of this sheet is enclosed.

XX If these papers are not considered timely filed by the Patent and Trademark Office, then a petition is hereby made under 37 C.F.R. §1.136, and any additional fees required under 37 C.F.R. §1.136 for any necessary extension of time may be charged to deposit Account No. 15-0030. A duplicate copy of this sheet is enclosed.

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*If the entry in Column 2 is less than the entry in Column 1 write "0" in Column 3.
**If the "Highest Number Previously paid for" IN THIS SPACE is less than 20 write "20" in this space.
***If the "Highest Number Previously paid for" IN THIS SPACE is less than 3 write "3" in this space.

7/93

0010-1075-0 PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :

HIROSHI MATSUI ET AL : ATTN: APPLICATION DIVISION

SERIAL NO: NEW APPLICATION :
(BASED ON PCT/JP98/03239)

FILED: HEREWITH :

FOR: METHOD FOR PRODUCING PURINE NUCLEOSIDE BY FERMENTATION

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows.

IN THE CLAIMS

Please amend the claims as follows.

Claim 6, lines 1-2, replace "any one of claims 3-5" with --claim 3--.

Claim 7, line 1, delete "or 4".

Claim 13, line 3, replace "any one of claims 1-12" with --claim 1--.

REMARKS

Claims 1-13 are active in this application.

Claims 6,7 and 13 have been amended to remove multiple dependencies. No new matter is believed to have been added to this application by these amendments.

Applicants submit that the present application is ready for examination on the merits. Early notice to this effect is earnestly solicited.

Respectfully submitted,

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METHOD FOR PRODUCING PURINE NUCLEOSIDE BY FERMENTATION

Technical Field

The present invention relates to a method for producing purine nucleosides such as inosine and guanosine which are important as raw materials for syntheses of 5'-inosinic acid and 5'-guanylic acid, respectively, and a novel microorganism used for the production.

Background Art

For the production of inosine and guanosine by fermentation, there have been known methods utilizing adenine auxotrophic strains or such strains further imparted with drug resistance against various drugs such as purine analogues, which strains belong to the genus *Bacillus* (Japanese Patent Publication Nos. 38-23039 (1963), 54-17033 (1979), 55-2956 (1980), and 55-45199 (1980), Japanese Patent Application Laid-Open No. 56-162998 (1981), Japanese Patent Publication Nos. 57-14160 (1982) and 57-41915 (1982), and Japanese Patent Application Laid-Open No. 59-42895 (1984)), or the genus *Brevibacterium* (Japanese Patent Publication Nos. 51-5075 (1976) and 58-17592 (1972), and Agric. Biol. Chem., 42, 399 (1978)) and the like.

Conventional acquisition of such mutant strains comprises subjecting microorganisms to a mutagenesis treatment such as UV irradiation and nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine) treatment and

selecting a desired strain by using a suitable selection medium. On the other hand, breeding of such mutant strains by the use of genetic engineering techniques have also been practiced for strains belonging to the genus *Bacillus* (Japanese Patent Application Laid-Open Nos. 58-158197 (1983), 58-175493 (1983), 59-28470 (1984), 60-156388 (1985), 1-27477 (1989), 1-174385 (1989), 3-58787 (1991), 3-164185 (1991), 5-84067 (1993), and 5-192164 (1993)) and the genus *Brevibacterium* (Japanese Patent Application Laid-Open No. 63-248394 (1988)).

Disclosure of Invention

The object of the present invention is to create a microorganism suitable for the production of the purine nucleoside by fermentation.

To achieve the aforementioned object, the present inventors conceived an idea of imparting purine nucleoside-producing ability to a bacterial strain of the genus *Escherichia*, which is different in the genus from microorganisms which have hitherto been used for the production of the purine nucleoside by fermentation, and successfully realized it. Thus, the present invention has been completed.

Thus, the present invention provides a microorganism belonging to the genus *Escherichia* and having purine nucleoside-producing ability.

Specifically, the present invention provides the

microorganism which has acquired the purine nucleoside-producing ability because of an increase of an activity of an enzyme involved in the purine nucleoside biosynthesis in cells of the microorganism. More specifically, the present invention provides the microorganism which has acquired the purine nucleoside-producing ability because of an increase of an expression amount of a gene for an enzyme involved in the purine nucleoside biosynthesis, and the microorganism which has acquired the purine nucleoside-producing ability because of deregulation of control of an enzyme involved in the purine nucleoside biosynthesis.

The enzyme involved in the purine nucleoside biosynthesis may be, for example, phosphoribosyl pyrophosphate (PRPP) amidotransferase and phosphoribosyl pyrophospahte (PRPP) synthetase.

As a means of desensitizing the control of an enzyme involved in purine nucleoside biosynthesis, for example, deficiency of a purine repressor can be mentioned.

The present invention further provides the microorganism which has acquired the purine nucleoside-producing ability because of blockage of a reaction branching from the purine nucleoside biosynthesis and leading to another metabolite.

Examples of the reaction branching from the purine nucleoside biosynthesis and leading to another metabolite include, for example, those catalyzed by an enzyme selected from succinyl-adenosine monophosphate (AMP) synthase,

purine nucleoside phosphorylase, adenosine deaminase, inosine-guanosine kinase, guanosine monophosphate (GMP) reductase, 5-phosphogluconate dehydrase, phosphoglucose isomerase, adenine deaminase, and xanthosine phosphorylase.

The present invention further provides the microorganism which is enhanced in the purine nucleoside-producing ability because of weakening of incorporation of a purine nucleoside into cells of the microorganism.

The incorporation of the purine nucleoside into cells of the microorganism may be weakened by blockage of a reaction involved in the incorporation of the purine nucleoside into cells of the microorganism. An example of the reaction involved in the incorporation of the purine nucleoside into cells of the microorganism is a reaction catalyzed by nucleoside permease.

The present invention further provides a method for producing a purine nucleoside by fermentation comprising culturing the aforementioned microorganism in a culture medium to produce and accumulate the purine nucleoside in the medium, and collecting the purine nucleoside.

The present invention described in details below.

(1) Microorganism belonging to the genus *Escherichia* and having purine nucleoside-producing ability

As examples of the microorganism belonging to the genus *Escherichia* used in the present invention, *Escherichia coli* (*E. coli*) and the like can be mentioned. When *E. coli* strains

are bred by genetic engineering techniques, *E. coli* K12 strain may be utilized.

The term "purine nucleoside" herein used include, for example, inosine, guanosine, and adenosine.

The term "purine nucleoside-producing ability" herein used means ability to produce and accumulate a purine nucleoside in a medium. The term "having purine nucleoside-producing ability" means that the microorganism belonging to the genus *Escherichia* produces and accumulates a purine nucleoside in a medium in a larger amount than a wild strain of *E. coli* such as W3110 strain, preferably means that the microorganism produces and accumulates inosine in a medium in an amount of not less than 50 mg/L, more preferably not less than 100 mg/L, still more preferably not less than 200 mg/L, most preferably not less than 500 mg/L under the condition described in Example 1 below.

In order to breed a microorganism belonging to the genus *Escherichia* and having purine nucleoside-producing ability, it may be adopted breeding by increasing an activity of an enzyme involved in the purine nucleoside biosynthesis in cells of the microorganism, for example, breeding by increasing an expression amount of a gene for the enzyme involved in the purine nucleoside biosynthesis. Alternatively, breeding by desensitizing control of an enzyme involved in the purine nucleoside biosynthesis may be adopted.

Furthermore, breeding by blocking a reaction branching

from purine nucleoside biosynthesis and leading to another metabolite and breeding by weakening of incorporation of a purine nucleoside into cells of the microorganism.

(2) Microorganism in which an activity of an enzyme involved in purine nucleoside biosynthesis in cells of the microorganism is increased

All the enzymes involved in the purine nucleoside biosynthesis and all the reactions catalyzed by those enzymes in microorganisms belonging to the genus *Escherichia* have already been elucidated (Escherichia coli and Salmonella, CELLULAR AND MOLECULAR BIOLOGY, Second Edition vol. 1 and vol. 2, ASM PRESS, WASHINGTON D.C.). The purine nucleoside-producing ability can be imparted by increasing an activity of an enzyme catalyzing a rate-limiting reaction among the enzymes. An example of the enzyme catalyzing a reaction of rate-limiting step is PRPP amidotransferase or PRPP synthetase.

Examples of means of increasing an activity of an enzyme involved in the purine nucleoside biosynthesis in cells are explained below but are not limited thereto.

As a means of increasing the activity of the enzyme involved in the purine nucleoside biosynthesis in cells, increasing an expression amount of the gene for the enzyme may be mentioned.

Examples of means of increasing the expression amount of the gene include improvement of a regulatory region of the gene and increase of a copy number of the gene, but are

not limited thereto.

The improvement of the regulatory region means making modification thereto to increase a transcription amount of a gene. For example, a promoter can be enhanced by, for example, introducing a mutation into the promoter to increase a transcription amount of a gene located downstream of the promoter. Besides introducing a mutation into a promoter, another promoter which functions in microorganisms such as lac, trp, tac, trc, and PL may be newly introduced. Further, an enhancer may be newly introduced to increase the transcription amount of the gene. Introduction of a gene such as a promoter into chromosome DNA is described in, for example, Japanese Patent Application Laid-Open No. 1-215280 (1989).

Specifically, the copy number of the gene may be increased by ligating a gene to a multi-copy vector to form a recombinant DNA, and allowing a microorganism to have the recombinant DNA. The vector includes widely used ones such as plasmids and phages, and, in addition to these, transposon (Berg, D.E. and Berg, C.M., Bio/Technol., 1, 417 (1983)) and Mu phage (Japanese Patent Application Laid-Open No. 2-109985 (1990)). It is also possible to integrate a gene into a chromosome by a method utilizing a plasmid for homologous recombination or the like to increase the copy number of the gene.

For breeding a microorganism belonging to the genus *Escherichia* and having an increased expression amount of a gene for an enzyme involved in the purine nucleoside

biosynthesis, necessary regions of genes may be obtained by amplified by PCR (polymerase chain reaction) mainly based on already available information about *E. coli* genes, and used for breeding of the microorganism.

For example, *purF*, which is a gene coding for PRPP amidotransferase, can be cloned from the chromosome DNA of *E. coli* K12 W3110 strain (ATCC27325) using a PCR technique. The chromosome DNA used for this may be derived from any strain of *E. coli*. The *purF* means a gene coding for PRPP amidotransferase, which is subjected to feedback inhibition by adenosine monophosphate (AMP) or guanosine monophosphate (GMP), and includes mutants generated due to genetic polymorphism and the like. Genetic polymorphism means a phenomenon that an amino acid sequences of a protein is partially altered due to naturally occurring mutation on the gene.

As a means of increasing the activity of the enzyme involved in the purine nucleoside biosynthesis in the cells, it is also possible to introduce a mutation into a structural gene of the enzyme to enhance the enzymatic activity of the enzyme itself.

As a means of increasing the activity of the enzyme involved in the purine nucleoside biosynthesis in the cells, it is also possible to desensitize control of the enzyme involved in the purine nucleoside biosynthesis.

The control of the enzyme involved in the purine nucleoside biosynthesis means a mechanism negatively

controlling the activity of the enzyme, and includes feedback inhibition by an intermediate in the biosynthesis pathway or a final product, attenuation, transcriptional suppression and the like. A purine nucleoside produced by a microorganism inhibits the activity of the enzyme involved in the purine nucleoside biosynthesis or represses expression of a gene encoding the enzyme through the control. Therefore, for allowing the microorganism to produce the purine nucleoside, it is preferable to desensitize the control.

The enzyme involved in the purine nucleoside biosynthesis, which undergoes the control, includes PRPP amidotransferase which is subjected to feedback inhibition by AMP or GMP and PRPP synthetase which is subjected to feedback inhibition by adenosine diphosphate (ADP). Besides, inosine monophosphate dehydrogenase (*guaB*) and GMP synthetase (*guaA*) are subjected to feedback inhibition by GMP. Also, a purine operon, *guaBA* is subjected to repression.

As a method for desensitizing the control, a method for introducing mutation into a gene encoding the enzyme or a regulatory region thereof may be mentioned. The mutation includes mutation desensitizing feedback inhibition, which is usually mutation in a structural gene. The mutation also includes mutation desensitizing attenuation, which is usually mutation in attenuator. The mutation also includes mutation desensitizing repression, which is usually mutation in a gene coding a regulatory protein which is called repressor, or mutation in an operator region.

The mutation desensitizing repression includes mutation inactivating a purine repressor. The purine repressor binds to an operator region of a purine operon under the condition that a purine nucleoside exists in a large amount, resulting in repression of transcription of the operon. Inactivation of the repressor leads to desensitization of the repression.

In order to introduce a mutation into a gene, the site-specific mutagenesis (Kramer, W. and Frits, H.J., Methods in Enzymology, 154, 350 (1987)), the recombinant PCR technique (PCR Technology, Stockton Press (1989)), chemical synthesis of a specific portion of DNA, hydroxylamine treatment of a gene of interest, treatment of microbial strains having a gene of interest by UV irradiation or a chemical agent such as nitrosoguanidine or nitrous acid and the like can be used. When function of a gene should be completely inactivated, addition or deletion of DNA may be introduced at a suitable restriction site.

A microorganism in which control of an enzyme involved in the purine nucleoside biosynthesis is desensitized can be selected by determining an expression amount of the enzyme through an enzymatic activity assay, or using antibodies. As an example of a method for obtaining a mutant strain in which control of an enzyme is desensitized, a method comprising selecting a strain growing in a minimal medium containing a purine analogue such as 8-azaadenine and 8-azaguanine, and determining change of an expression amount

or activity of the enzyme.

(3) Microorganism which has acquired the purine nucleoside-producing ability because of blockage of a reaction branching from purine nucleoside biosynthesis and leading to another metabolite

The purine nucleoside biosynthesis pathway of microorganisms belonging to the genus *Escherichia* has been already elucidated, and all the enzymes involved in the purine nucleoside biosynthesis and reactions catalyzed by those enzymes have also been elucidated (Escherichia coli and Salmonella, CELLULAR AND MOLECULAR BIOLOGY, Second Edition, vol. 1 and vol. 2, ASM PRESS WASHINGTON D.C.). In addition, some of reactions which lead to other metabolites have also been made clear.

A microorganism in which a reaction leading to another metabolite are blocked may become to require the metabolite. In order to cultivate such microorganism that has become to require the metabolite, it is necessary to add the metabolite or an intermediate (precursor) therefor to a culture medium as a nutrient. Therefore, it is desirable that a reaction not requiring extra addition of the metabolites when it is blocked should be selected as the reaction to be blocked.

The purine nucleoside-producing ability may not be always improved by blocking any of the reactions leading to other metabolites. If a reaction converting a purine nucleoside intermediate or a purine nucleoside into another metabolite proceeds during the production of the purine

nucleoside by the microorganism, blocking such a reaction may improve the purine nucleoside productivity.

A reaction whose blocking may actually improve the purine nucleoside-producing ability may be predicted among the reactions branching from the purine nucleoside biosynthesis and leading to the production of another metabolite based on the already elucidated schemes of the purine nucleoside biosynthesis.

As a method for blocking the reaction branching from the purine nucleoside biosynthesis and leading to another metabolite, a method for deleting or inactivating an enzyme catalyzing the reaction or the like may be mentioned. The enzyme may be deleted, for example, by deleting a gene encoding the enzyme. The enzyme may be inactivated by, for example, introducing a mutation into a gene encoding the enzyme, adding an agent specifically inactivating the enzyme or the like.

Examples of the reaction branching from the purine nucleoside biosynthesis and leading to another metabolite, whose blocking may actually improve the purine nucleoside-producing ability, include a reaction catalyzed by an enzyme selected from succinyl-AMP synthase, purine nucleoside phosphorylase, adenosine deaminase, inosine-guanosine kinase, GMP reductase, 6-phosphogluconate dehydrase, phosphoglucose isomerase, adenine deaminase, and xanthosine phosphorylase.

For example, when the branching from IMP to

succinyl-AMP and the conversion from inosine to hypoxanthine are blocked, IMP is not converted to AMP and inosine is not converted to hypoxanthine. Accordingly, it is expected that inosine is accumulated. In order to evaluate the effectiveness of such blocking, a mutant obtained depending on the purpose may be cultured, and its inosine productivity may be determined.

As described in the Examples hereinafter, when *E. coli* was made adenine auxotrophic by destroying succinyl-AMP synthase gene (*purA* gene), it became necessary to add an AMP substance such as adenine and adenosine to a culture medium for the growth of the adenine auxotroph of *E. coli*. However, it was found in *E. coli* that such an added substance was immediately converted to inosine or hypoxanthine, and its growth was ceased at a certain point due to the loss of the AMP substance. Therefore, judging from the metabolic pathway of *E. coli*, it is expected that it is necessary to inactivate adenosine deaminase involved in the conversion of adenosine to inosine or adenine deaminase involved in the conversion of adenine to hypoxanthine as a means of maintaining its growth. Thus, the effectiveness of the inactivation of adenosine deaminase or adenine deaminase was confirmed, and accumulation of inosine was observed.

GMP reductase is involved in the conversion of GMP to IMP. It is expected that guanosine productivity is improved by inactivating the GMP reductase. As shown in Examples below, a certain level of improvement in guanosine accumulation was

observed.

A carbon source such as glucose is used for the production of a purine nucleoside. It is known that there is a difference in sugar metabolic system leading to purine nucleoside biosynthesis depending on the used carbon source or culture conditions. Therefore, for leading the metabolic system to purine nucleoside biosynthesis advantageously, it is considered to block branches other than pentose phosphate pathway to give preference in the pentose phosphate pathway. As a means thereof, inactivation of 6-phosphogluconate dehydrase or phosphoglucose isomerase was tested and the effectiveness thereof was confirmed.

(4) Microorganism which has acquired the purine nucleoside-producing ability by weakening of incorporation of a purine nucleoside into cells of the microorganism

Since incorporation of a purine nucleoside which has released externally from cells into the cells again is considered unreasonable in view of energy for accumulating the purine nucleoside, weakening of incorporation of a purine nucleoside is effective.

As means of weakening incorporation of a purine nucleoside into cells, blocking of a reaction involved in membrane permeability of the purine nucleoside may be mentioned. The blocking of the reaction can be carried out in the same manner as described about (3) above.

For example, by inactivating nucleoside permease which is one of permeases involved in incorporation of purine

nucleosides into cells, improvement in accumulation of inosine was observed.

(5) Method for producing a purine nucleoside

The method for producing a purine nucleoside by fermentation using the microorganism having the purine nucleoside-producing ability is explained hereinafter.

Culture medium for purine nucleoside production to be used may be a usual medium containing a carbon source, a nitrogen source, inorganic ions and other organic components as required. As the carbon source, saccharides such as glucose, lactose, galactose, fructose, arabinose, maltose, xylose, trehalose, ribose and hydrolysates of starches; alcohols such as glycerol, mannitol and sorbitol; organic acids such as gluconic acid, fumaric acid, citric acid and succinic acid and the like can be used. As the nitrogen source, inorganic ammonium salts such as ammonium sulfate, ammonium chloride, and ammonium phosphate; organic nitrogen such as of soy bean hydrolysates; ammonia gas; aqueous ammonia and the like can be used. It is desirable that vitamins such as vitamin B1, required substances, for example, nucleic acids such as adenine and RNA, or yeast extract and the like are contained in appropriate amounts as trace amount organic nutrients. Other than these, small amounts of calcium phosphate, magnesium sulfate, iron ions, manganese ions and the like may be added, if necessary.

Cultivation is preferably performed under an aerobic condition for 16 to 72 hours, and culture temperature during

the cultivation is controlled within 30 to 45°C and pH within 5 to 8. The pH can be adjusted by using an inorganic or organic acidic or alkaline substance as well as ammonia gas.

A purine nucleoside can be recovered from the fermentation liquor by any or any combination of conventional methods such as techniques utilizing ion exchange resin and precipitation.

(6) Specific examples of purine nucleoside-producing bacteria

First, *purF* (a gene coding for PRPP amidotransferase), *purR* (a gene coding for a purine repressor), *deoD* (a gene coding for purine nucleoside phosphorylase), *purA* (a gene coding for succinyl-AMP synthase), *add* (a gene coding for adenosine deaminase), *gsk* (a gene coding for inosine-guanosine kinase), *guaC* (a gene coding for GMP reductase), *edd* (a gene coding for 6-phosphogluconate dehydrase), *pgi* (a gene coding for phosphoglucose isomerase), *yicP* (a gene for coding for adenine deaminase), *prs* (a gene coding for PRPP synthetase), *xapA* (a gene coding for xanthosine phosphorylase), and *nupG* (a gene coding for nucleoside permease) are cloned from a chromosome DNA of *Escherichia coli* (*E. coli*) K12 strain W3110 (ATCC27325) by using a PCR technique, and they may be mutated depending on their purposes. The chromosome DNA used for this procedure may be obtained from any strain of *E. coli*.

The mutation introduced into *purF* is a mutation for destroying *purF* or a mutation for desensitizing the feedback

inhibition of PRPP amidotransferase. The mutation introduced into *purR* is a mutation for destroying *purR*. The mutation introduced into *deoD* is a mutation for destroying *deoD*. The mutation introduced into *purA* is a mutation for destroying *purA*. The mutation introduced into *add* is a mutation for destroying *add*. The mutation introduced into *gsk* is a mutation for destroying *gsk*.

The mutation introduced into *guaC* is a mutation for destroying *guaC*. The mutation introduced into *edd* is a mutation for destroying *edd*. The mutation introduced into *pgi* is a mutation for destroying *pgi*. The mutation introduced into *yicP* is a mutation for destroying *yicP*. The mutation introduced into *prs* is a mutation for desensitizing the feedback inhibition of PRPP synthetase. The mutation introduced into *xapA* is a mutation for destroying *xapA*. The mutation introduced into *nupG* is a mutation for destroying *nupG*.

To introduce a mutation into a gene, the site-specific mutagenesis (Kramer, W. and Frits, H.J., Methods in Enzymology, 154, 350 (1987)), the recombinant PCR technique (PCR Technology, Stockton Press (1989)), chemical synthesis of a specific portion of DNA, hydroxylamine treatment of a gene of interest, treatment of microbial strains having a gene of interest by UV irradiation or a chemical agent such as nitrosoguanidine or nitrous acid and the like can be used. When function of a gene should be completely inactivated, addition or deletion of DNA may be introduced at a suitable

restriction site.

Then, *purF* and *prs* to which a mutation for desensitizing the feedback inhibition of PRPP amidotransferase and PRPP synthetase are added, respectively, are introduced as a recombinant DNA into a suitable microorganism to express the genes, thereby obtaining a microorganism containing the PRPP amidotransferase gene (*purF*) and the PRPP synthetase gene (*prs*) whose feedback inhibition is substantially desensitized. The recombinant DNA obtained above means a vector such as plasmid and phage, into which a useful gene such as the PRPP amidotransferase gene (*purF*) and the PRPP synthetase (*prs*) whose feedback inhibition is substantially desensitized is integrated as a passenger. The vector may contain a promoter operable in the microorganism, such as lac, trp, tac, trc, and PL so that efficient expression of the useful gene can be obtained.

The recombinant DNA herein used includes any of those obtained by integrating a useful gene into a chromosome by using a transposon (Berg, D.E. and Berg, C.M., Bio/Technol., 1, 417(1983)), Mu phage (Japanese Patent Application Laid-Open No. 2-109985 (1990)), a plasmid for homologous recombination or the like.

As the plasmid for homologous recombination, a plasmid having a temperature-sensitive replication origin may be used. The plasmid having the temperature-sensitive replication origin can replicate at a permissive temperature, for example, around 30°C, but cannot replicate at a non-permissive

temperature, for example, 37°C to 42°C. In the homologous recombination method using the plasmid having the temperature-sensitive replication origin, the plasmid can be replicated at a permissive temperature, or dropped out at a non-permissive temperature as required. In the Examples described below, pMAN997, which corresponds to pMAN031 (J. Bacteriol., 162, 1196 (1985)) whose *Vsp*I-*Hin*dIII fragment is replaced with that of pUC19 (Takara Shuzo) (Figure 1), was used as the plasmid for homologous recombination.

A specific genetic function on the chromosome was inactivated by the homologous recombination (Experiments in Molecular Genetics, Cold Spring Habor Lab. (1972)) to improve the purine nucleoside-producing ability. The gene to be inactivated is a gene of which inactivation leads increase of an expression amount of a gene for an enzyme involved in the purine nucleoside biosynthesis. Specifically, the purine repressor gene (*purR*) on the chromosome was destroyed to remove the expression regulation mechanism of the purine nucleotide biosynthesis genes including the PRPP amidotransferase gene (*purF*).

Further, a gene coding for an enzyme which catalyzes a reaction branching from purine nucleoside biosynthesis and leading to another metabolite was destroyed. Specifically, the purine nucleoside phosphorylase gene (*deoD*) was destroyed to suppress the decomposition of inosine and guanosine to hypoxanthine and guanine, respectively. Furthermore, the succinyl-AMP synthase gene (*purA*) was destroyed to impart

adenine auxotrophy. Moreover, the adenosine deaminase gene (*add*) was destroyed to suppress the conversion of adenosine to inosine. Finally, the inosine-guanosine kinase gene (*gsk*) was destroyed to suppress the conversion of inosine and guanosine to IMP and GMP, respectively. The GMP reductase gene (*guaC*) was destroyed to suppress the conversion of GMP to IMP. The 6-phosphogluconate dehydrase gene (*edd*) was destroyed to suppress metabolism of sugars through the Entner-Doudoroff pathway. The phosphoglucose isomerase gene (*pgi*) was destroyed to suppress metabolism of sugars through glycolysis pathway, thereby promoting the flow into the pentose phosphate pathway. The adenine deaminase gene (*yicP*) was destroyed to suppress the conversion of adenine to hypoxanthine. The xantosine phosphorylase gene (*xapA*) was destroyed to suppress the decomposition of xanthosine to xanthine and to suppress the decomposition of inosine and guanosine to hypoxanthine and guanine, respectively. The inactivation of a target gene may also be performed of course by treatment of microbial strains having the genes with UV irradiation or with a chemical agent such as nitrosoguanidine and nitrous acid.

As the microorganism having the recombinant DNA, a microorganism belonging to the genus *Escherichia* in which a gene coding for a target enzyme such as PRPP amidotransferase was expressed was used.

In order to efficiently utilize the PRPP amidotransferase gene (*purF*), it is preferably used together

with other useful genes, for example, genes involved in the IMP biosynthesis from PRPP other than *purF* (*purD*, *purT*, *purL*, *purM*, *purK*, *purE*, *purC*, *purB*, *purH*), IMP dehydrogenase gene (*guaB*), GMP synthetase gene (*guaA*), PRPP synthetase gene (*prs*) and the like. Like the PRPP amidotransferase gene (*purF*), those useful genes may be present on a host chromosome, or a plasmid or phage.

A microorganism having deficiency of *purA* (succinyl-AMP synthase gene) and/or deficiency of *deoD* (purine nucleoside phosphorylase gene) and/or deficiency of *purR* (purine repressor gene) and/or desensitized type *purF* (PRPP amidotransferase gene) and/or deficiency of *add* (adenosine deaminase gene) and/or deficiency of *gsk* (inosine-guanosine kinase gene) and/or deficiency of *guaC* (GMP reductase gene) and/or deficiency of *edd* (6-phosphogluconate dehydrase gene) and/or deficiency of *pgi* (phosphoglucose isomerase gene) and/or deficiency of *yicP* (adenine deaminase gene) and/or deficiency of *xapA* (xanthosine phosphorylase gene) and/or deficiency of *nupG* (nucleoside permease gene), or a microorganism transformed with a recombinant DNA having desensitized type PRPP amidotransferase gene (*purF*) and/or desensitized type *prs* (PRPP synthetase gene) obtained as described above is cultured so that the target purine nucleoside such as inosine and guanosine is accumulated in the culture medium, and the accumulated nucleoside is collected.

Brief Description of Drawings

Figure 1 shows a construction of pMAN997.

Figure 2 shows structures of genes for homologous recombination. Numerals in the figure represent lengths (bp) of obtained fragments and positions from 5' ends.

Figure 3 shows structures of genes for homologous recombination. Numerals in the figure represent lengths (bp) of obtained fragments and positions from 5' ends.

Best Mode for Carrying Out the Invention

Example 1

1) Breeding of strain deficient in PRPP amidotransferase gene (*purF*)

PCR was carried out (94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using chromosome DNA of E. coli K12 strain W3110 (ATCC27325) as a template and 29-mer and 31-mer primers for both ends, having nucleotide sequences of CTCCTGCAGAACGAGGAAAAAGACGTATG (SEQ ID NO: 1) and CTCAAGCTTTCATCCTTCGTTATGCATTTCG (SEQ ID NO: 2), and prepared based on the information of a gene data bank (GenBank Accession No. M26893), and an amplified fragment of about 1530 bp of the *purF* structural gene region covering SD-ATG and the translation termination codon was cloned into pCRTMII vector (Invitrogen). The amplified fragment of the PCR product can be cloned into this vector as it is. The vector has *Eco*RI sites as restriction sites at vicinities of the

both sides of the cloning site. A *Pst*I site and a *Hin*dIII site are respectively provided in the PCR primers.

The cloned 1530 bp *purF* fragment contained one *Bgl*II site at about 880 bp from the 5' end, and pCRTMII vector itself also had one *Bgl*II site. Therefore, the plasmid was partially digested with *Bgl*II, blunt-ended by T4 DNA polymerase, and then ligated by T4 DNA ligase. Competent cells of *E. coli* HB101 were transformed with this ligation solution, and transformants grown on LB (1% tryptone, 0.5% yeast extract, 0.1% NaCl, 0.1% Glucose, pH 7) agar plates containing 25 μg/ml of ampicillin were obtained. Plasmid DNAs were prepared from the transformants of 18 clones, and a plasmid DNA which provided a fragment of about 1550 bp by the *Eco*RI digestion, which fragment was not digested with *Bgl*II (pCRTMIIpurF'#14) was selected from the plasmid DNAs. The *purF* contained in this plasmid DNA has a frame shift at the *Bgl*II site, and therefore it is predicted that the encoded enzyme lacks its function (Figure 2).

Then, the pCRTMIIpurF'#14 was digested with *Eco*RI to prepare a fragment of about 1.6 Kb that included the *purF*. This fragment was inserted into the *Eco*RI site of pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (As shown in Figure 1, pMAN031 (J. Bacteriol., 162, 1196(1985)) of which *Vsp*I-*Hin*dIII fragment is replaced with that of pUC19 (Takara Shuzo)), to obtain plasmid pMAN997purF'#14. *E. coli* W3110 (wild type) was transformed at 30°C with the pMAN997purF'#14,

and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin, and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture broth was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. Among the ampicillin-sensitive clones, clones that were not grown in a minimal medium (Na_2HPO_4 6.8 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2 \cdot 2H_2O$ 15 mg, thiamin·HCl 2 mg, glucose 0.2 g per 1 L), but grown in the minimal medium supplemented with 50 mg/L of hypoxanthine were further selected. Furthermore, the fragment of about 1.5 kb

including *purF* was amplified by PCR from the chromosome DNA of the above obtained target clones, and confirmed not to be digested with *Bgl*II. The clones satisfying the above conditions were considered strains deficient in *purF*, and designated as strains F-2-51 and F-1-72.

2) Breeding of strain deficient in succinyl-AMP synthase gene (*purA*)

PCR was carried out (94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 as a template and 31-mer primers for both ends, having nucleotide sequences of CTCGAGCTCATGGGTAACAACGTCGTCGTAC (SEQ ID NO: 3) and CTCGTCGACTTACGCGTCGAACGGGTCGCGC (SEQ ID NO: 4), and prepared based on the information of a gene data bank (GenBank Accession No. J04199), and an amplified fragment of about 1300 bp of the *purA* structural gene region covering ATG and the translation termination codon was cloned between the *Sac*I and *Sal*I sites of pUC18 vector (Takara Shuzo). A *Sac*I site and a *Sal*I site are respectively provided in the PCR primers. The cloned *purA* fragment of about 1300 bp contained one *Hpa*I site and one *Sna*BI site respectively at about 520 bp and 710 bp from the 5' end, and therefore the plasmid was digested with *Hpa*I and *Sna*BI, and ligated by T4 DNA ligase to obtain the plasmid from which a fragment of about 190 bp was removed. Competent cells of *E. coli* JM109 were transformed with this ligation solution, and transformants grown on LB agar plates

containing 25 μg/ml of ampicillin were obtained. Plasmid DNAs were prepared from the transformants of 18 clones, and a plasmid DNA that was not digested with *Fsp*I but provided a fragment of about 1100 bp fragment by *Sac*I and *Sal*I digestion (pUC18purA'#1) was selected from the plasmid DNAs. The *purA* contained in this plasmid DNA has a deletion between the *Hpa*I and *Sna*BI sites, and therefore it is predicted that the encoded enzyme lacks its function (Figure 2).

Then, the pUC18purA'#1 was digested with *Sac*I and *Sal*I to prepare a fragment of about 1.1 kb that included the *purA*. This fragment was inserted between the *Sac*I and *Sal*I sites of pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (described above), to obtain plasmid pMAN997purA'#1. The strain F-2-51 (*purF*⁻) was transformed at 30°C with the plasmid pMAN997purA'#1, and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin, and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then

inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture broth was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. Among the ampicillin-sensitive clones, clones that were not grown on the minimal medium supplemented with 50 mg/L of hypoxanthine, but grown on the minimal medium supplemented with 50 mg/L of adenine were further selected. Furthermore, the *purA* fragment of about 1.1 kb was amplified by PCR from the chromosome DNA of these target clones, and confirmed to be smaller than the wild type (about 1.3 kb) and not to be digested with *Fsp*I. The clone satisfying the above conditions was considered a strain deficient in *purA* , and designated as strain FA-31.

3) Breeding of strain deficient in purine nucleoside phosphorylase gene (*deoD*)

PCR was carried out (94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 as a template and 30-mer and 31-mer primers for both ends, having nucleotide sequences of CTCGTCGACGCGGGTCTGGAACTGTTCGAC (SEQ ID NO: 5)

and CTCGCATGCCCGTGCTTTACCAAAGCGAATC (SEQ ID NO: 6), and prepared based on the information obtained through searching of a gene data bank (*E. coli* Gene Bank) using "deoD" as a key word, and an amplified fragment of about 1350 bp including a *deoD* structural gene region covering SD-ATG and the translation termination codon was cloned into pCRTMII vector (Invitrogen). The vector has *Eco*RI sites as restriction sites at vicinities of the both sides of the cloning site. A *Sal*I site and a *Sph*I site are respectively provided in the PCR primers. The cloned *deoD* fragment of about 1350 bp contained one *Hpa*I site at about 680 bp from the 5' end, and therefore the plasmid was digested with *Hpa*I, and a mixture of the digested plasmid and a 10-mer *Cla*I linker was subjected to T4 DNA ligase reaction. As a result, a *Cla*I site was inserted at the *Hpa*I site. Competent cells of *E. coli* HB101 were transformed with this ligation solution, and transformants grown on LB agar plates containing 25 μg/ml of ampicillin were obtained. Plasmid DNAs were prepared from the transformants of 16 clones, and a plasmid DNA that was not digested with *Hpa*I but digested with *Cla*I (pCRTMIIdeoD'#16) was selected from the plasmid DNAs. The *deoD* contained in this plasmid has a frame shift at the *Hpa*I site, and therefore it is predicted that the encoded enzyme lacks its function (Figure 2).

Then, the pCRTMIIdeoD'#16 was digested with *Eco*RI to prepare a fragment of about 1.35 kb that included the *deoD*. This fragment was inserted into the *Eco*RI site of pMAN997,

which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (described above), to obtain plasmid pMAN997deoD'#16. The strain F-1-72 (*purF*⁻) and the strain FA-31 (*purF*⁻, *purA*⁻) were transformed at 30°C with plasmid pMAN997deoD'#16, and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin, and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture broth was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. The ampicillin-sensitive clones were allowed to

grow on the LB medium supplemented with 1 g/L of inosine, and clones that did not decompose inosine to hypoxanthine were selected through thin layer chromatography analysis of the culture medium. Furthermore, the fragment of about 1.35 kb including *deoD* was amplified by PCR from the chromosome DNA of these target clones, and confirmed to be digested with *Cla*I but not to be digested with *Hpa*I. The clones satisfying the above conditions were considered strains deficient in *deoD*, and clones derived from the strain F-1-72 (*purF*$^-$) and the strain FA-31 (*purF*$^-$, *purA*$^-$) were designated as strains FD-6 and FAD-25, respectively.

4) Breeding of strain deficient in purine repressor gene (*purR*)

PCR was carried out (94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 as a template and 29-mer and 28-mer primers for both ends, having nucleotide sequences of CTCGTCGACGAAAGTAGAAGCGTCATCAG (SEQ ID NO: 7) and CTCGCATGCTTAACGACGATAGTCGCGG (SEQ ID NO: 8), and prepared based on the information obtained through searching of a gene data bank (*E. coli* Gene Bank) using "purR" as a key word, and an amplified fragment of about 1.8 kb including a *purR* structural gene region covering ATG and the translation termination codon and about 800 bp 5' upstream region of ATG was cloned between the *Sal*I site and the *Sph*I site of pUC19 vector (Takara Shuzo). A *Sal*I site and a *Sph*I

site are respectively provided in the PCR primers, and these sites are used for cloning. The cloned *purR* fragment of about 1.8 kb contained one *Pma*CI site at about 810 bp from the 5' end (vicinity of N-terminus in the *purR* structural gene region), and therefore the plasmid was digested with *Pma*CI. A mixture of the digested plasmid and a 8-mer *Bgl*II linker was subjected to T4 DNA ligase reaction. As a result, a *Bgl*II site was inserted at the *Pma*CI site. Competent cells of *E. coli* JM109 were transformed with this ligation solution, and transformants grown on LB agar plates containing 25 μg/ml of ampicillin were obtained. Plasmid DNAs were prepared from the transformants of 10 clones, and a plasmid DNA not digested with *Pma*CI but digested with *Bgl*II (pUC19purR'#2) was selected from the plasmid DNAs. The *purR* contained in this plasmid DNA has a frame shift at the *Pma*CI site, and therefore it is predicted that the encoded enzyme lacks its function (Figure 2).

Then, the pUC19purR'#2 was digested with *Sac*I and *Sph*I to prepare a fragment of about 1.8 kb that included the *purR*. This fragment was inserted between the *Sac*I site and the *Sph*I site of pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (described above), to obtain plasmid pMAN997purR'#2. The strain FD-6 (*purF*$^-$, *deoD*$^-$) and the strain FAD-25 (*purF*$^-$, *purA*$^-$, *deoD*$^-$) were transformed at 30°C with the plasmid pMAN997purR'#2, and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of

ampicillin, and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture broth was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. Ten clones were randomly selected from the ampicillin-sensitive clones, and the fragment of about 1.8 kb including *purR* was amplified by PCR from the chromosome DNA of these clones, and clones that were digested with *Bgl*II but not with *Pma*CI were selected. These clones were considered strains deficient in *purR*, and clones derived from the strain FD-6 (*purF*$^-$, *deoD*$^-$) and the strain FAD-25 (*purF*$^-$,

purA$^-$, *deoD*$^-$) are designated as strain FDR-18 and strain FADR-8, respectively. It was confirmed that the PRPP amidotransferase activity in the strains in which *purR* was destroyed was increased compared with that of a strain in which *purR* was not destroyed by using the *purF*$^+$ strain deficient in *deoD* and *purR* or the *purF*$^+$ strain deficient in *purA*, *deoD* and *purR*. The PRPP amidotransferase activity was measured according to the method of L. J. Messenger et al. (J. Biol. Chem., 254, 3382(1979)).

5) Construction of desensitized type PRPP amidotransferase gene (*purF*)

A *purF* fragment was excised from the plasmid carrying the *purF* of about 1530 bp cloned into pCRTMII vector (Invitrogen) in Section 1) by digestion with *Pst*I and *Hin*dIII, and inserted between the *Pst*I and *Hin*dIII sites of the multi-cloning site of a plasmid for introducing mutation, pKF18 (Takara Shuzo) to obtain the target clone (pKFpurF). G. Zhou et al. (J. Biol. Chem., 269, 6784 (1994)) has revealed that PRPP amidotransferase (PurF) whose Lys (K) at position 326 is replaced with Gln (Q) and the same whose Pro (P) at position 410 is further replaced with Trp (W) are each desensitized for feedback inhibition by GMP and AMP. Therefore, the following synthetic DNA primers were prepared for gene substitution realizing mutations of Lys (K) at position 326 and Pro (P) at position 410 of PRPP amidotransferase (PurF) to Gln (Q) and Trp (W), respectively,

and pKFpurF was subjected to site-directed mutagenesis according to the protocol of Site-directed Mutagenesis System Mutan-Super Express Km (Takara Shuzo) to introduce a site-directed mutation into the pKFpurF.

Primer for K326Q mutation:

5'-GGGCTTCGTT CAG AACCGCTATGTTGG-3' (SEQ ID NO: 9)

Primer for P410W mutation:

5'-TATGGTATTGATATG TGG AGCGCCACGGAAC-3' (SEQ ID NO: 10)

After the mutagenesis, 6 clones were randomly picked up from each of the resulting transformants, and plasmids were prepared from them. By nucleotide sequencing of the plasmids around the locations where the mutations were introduced, it was confirmed that target mutants were obtained. The obtained plasmids were designated as pKFpurFKQ and pKFpurFPW, respectively. The mutation P410W (410Pro→Trp) was further introduced into pKFpurFKQ in the same manner to prepare pKFpurFKQPW, a mutant plasmid having two mutations simultaneously. Each of the plasmids pKFpurFKQ, pKFpurFPW and pKFpurFKQPW has an inserted mutant *purF* downstream of the lacp/o (promoter of lactose operon) derived from pKF18, and the *purF* is expressed under the control of this promoter.

Recombinant bacteria obtained by transforming *E. coli* JM109 cells with the above plasmids were cultured in LB liquid medium for eight hours, and collected, and crude enzyme extracts were prepared from them. The PRPP amidotransferase activity of the extracts and degrees of inhibition by AMP

and GMP were measured according to the method of L. J. Messenger (J. Biol. Chem., 254, 3382 (1979)). The results are shown in Table 1.

Table 1

PRPP amidotransferase activity and inhibition by AMP and GMP

Host	Plasmid	PRPP amidotransferase activity (μmole/min/mg)		
		None	10 mM AMP	10 mM GMP
JM109	-	0.001	-	-
JM109	pKFpurF	0.68	0.48	0.10
JM109	pKFpurFKQ	0.34	0.32	0.33
JM109	pKFpurFKQPW	0.18	0.16	0.17

6) Evaluation of purine nucleoside-producing ability of mutant *purF* plasmid-introduced strain

Transformants were produced by introducing pKFpurFKQ and pKFpurFKQPW into the strain FDR-18 (*purF*$^-$, *deoD*$^-$, *purR*$^-$) and the strain FADR-8 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$) produced in Section 4), and purine nucleoside-producing abilities of these strains were evaluated.

Basal medium and culture method for purine nucleoside production and analysis method for the evaluation of the purine nucleoside-producing ability will be described below.

1. Basal medium: MS medium

	Final concentration
Glucose	40 g/L (separately sterilized)

$(NH_4)_2SO_4$	16 g/L
KH_2PO_4	1 g/L
$MgSO_4 \cdot 7H_2O$	1 g/L
$FeSO_4 \cdot 7H_2O$	0.01 g/L
$MnSO_4 \cdot 4H_2O$	0.01 g/L
Yeast extract	2 g/L
$CaCO_3$	30 g/L (separately sterilized)

2. Culture method

Refresh culture; a stored bacterium inoculated

LB agar medium (supplemented with a drug as required)

37°C, cultured overnight

Seed culture; the refresh cultured bacterium inoculated

LB liquid medium (supplemented with a drug as required)

37°C, cultured overnight

Main culture; 2% inoculated from the seed culture

MS medium (supplemented with adenine and a drug as required)

37°C, 20 ml/500-ml volume Sakaguchi's culture flask

3. Analysis method

A sample of the culture medium (500 μl) is repeatedly taken in the time course, and centrifuged at 15,000 rpm for 5 minutes, and the supernatant is diluted 4 times with H_2O and analyzed by HPLC. Unless noted otherwise, the evaluation is made based on an accumulated amount of a purine nucleoside per unit volume of the medium after culture for 3 days.

Analysis conditions:

Column: Asahipak GS-220 (7.6 mm ID x 500 mm L)

Buffer: pH is adjusted with 0.2M NaH_2PO_4 (pH 3.98), and phosphoric acid

Temperature : 55°C

Flow Rate: 1.5 ml/min

Detection: UV 254 nm

Retention time (min)

Inosine	16.40
Hypoxanthine	19.27
Guanosine	20.94
Guanine	23.55
Adenine	24.92
Adenosine	26.75

For the strains of *purA*$^-$ (adenine auxotrophic), 5 mg/L of adenine was added to the MS medium.

The results of the evaluation of the purine nucleoside-producing ability are shown in Table 2. Superior inosine production was observed with respect to the mutant *purF* plasmid-introduced strains in contrast to the strain W3110 (wild type strain) by which a trace amount of the production was observed.

Table 2

Evaluation of purine nucleoside-producing ability

Host	Plasmid	Purine nucleoside accumulation	
		Inosine (mg/L)	Guanosine (mg/L)
W3110	-	Trace	0
FDR-18	pKFpurFKQ	115	0
FDR-18	pKFpurFKQPW	110	0
FADR-8	pKFpurFKQ	66	0
FADR-8	pKFpurFKQPW	62	0

Example 2

1) Breeding of strain deficient in adenosine deaminase gene (*add*)

PCR was carried out(94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 as a template and 29-mer primers for both ends, having nucleotide sequences of CTCGTCGACGGCTGGATGCCTTACGCATC (SEQ ID NO: 11) and CTCGCATGCAGTCAGCACGGTATATCGTG (SEQ ID NO: 12), and prepared based on the information obtained through searching of a gene data bank (*E. coli* Gene Bank) using "add" as a key word, and an amplified fragment of about 1.8 kb including an *add* structural gene region covering ATG and the translation termination codon, about 420 bp 5′ upstream region of ATG and about 370 bp downstream region of the translation termination codon was cloned between the *Sal*I site and the *Sph*I site of pUC19 vector (Takara Shuzo). A *Sal*I site and

a *Sph*I site are respectively provided in the PCR primers, and these sites are used for the cloning. The cloned *add* fragment of about 1.8 kb contained one *Stu*I site at about 880 bp from the 5' end, and therefore the plasmid was digested with *Stu*I, and a mixture of the digested plasmid and a 8-mer *Bgl*II linker was subjected to T4 DNA ligase reaction. As a result, a *Bgl*II site was inserted at the *Stu*I site. Competent cells of *E. coli* JM109 were transformed with this ligation solution, and transformants grown on LB agar plates containing 25 μg/ml of ampicillin were obtained. Plasmid DNAs were prepared from the transformants of 10 clones, and a plasmid DNA not digested with *Stu*I but digested with *Bgl*II (pUC19add'#1) was selected from the plasmid DNAs. The *add* contained in this plasmid DNA has a frame shift at the *Stu*I site, and therefore it is predicted that the encoded enzyme lacks its function (Figure 2).

Then, the pUC19add'#1 was digested with *Sac*I and *Sph*I to prepare a fragment of about 1.8 kb that included the *add*. This fragment was inserted between the *Sac*I site and the *Sph*I site of pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (described above), to obtain plasmid pMAN997add'#1. The strain FDR-18 (*purF*$^-$, *deoD*$^-$, *purR*$^-$) and the strain FADR-8 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$) were transformed at 30°C with the plasmid pMAN997add'#1, and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin, and cultured at 30°C overnight. Then,

the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture broth was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. The ampicillin-sensitive clones were allowed to grow in the LB medium supplemented with 1.5 g/L of adenosine, and clones that did not convert adenosine to inosine were selected through thin layer chromatography analysis of the culture medium. Furthermore, the *add* fragment of about 1.8 kb was amplified by PCR from the chromosome DNA of these target clones, and confirmed to be digested with *Bgl*II but not to be digested with *Stu*I. These clones were considered strains

deficient in *add*, and clones derived from the strain FDR-18 (*purF*$^-$, *deoD*$^-$, *purR*$^-$) and the strain FADR-8 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$) are designated as strains FDRadd-18-1 and FADRadd-8-3, respectively.

2) Evaluation of purine nucleoside-producing ability of desensitized type *purF*-introduced strain

Transformants were made by introducing pKFpurFKQ and pKFpurFKQPW into the strain FDRadd-18-1 (*purF*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) and the strain FADRadd-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) bred in Section 1), and purine nucleoside-producing abilities of these strains were evaluated. For the strain FADRadd-8-3, a transformant with the wild type *purF* plasmid (pKFpurF) was also made, and compared with the transformant with pKFpurFKQ and the transformant with pKFpurFKQPW. The basal medium and the culture method for the purine nucleoside production and the analysis method were the same as Example 1.

The results of the evaluation of the purine nucleoside-producing ability are shown in Table 3. Superior inosine production was observed compared with the strain W3110 (wild type strain). Effects of desensitized type *purFKQ* and *purFKQPW* were observed by comparing with the wild type *purF*.

Table 3

Evaluation of purine nucleoside-producing ability

Host	Plasmid	Purine nucleoside accumulation	
		Inosine (mg/L)	Guanosine (mg/L)
W3110	-	Trace	0
FDRadd-18-1	pKFpurFKQ	220	0
FDRadd-18-1	pKFpurFKQPW	215	0
FADRadd-8-3	pKFpurFKQ	1080	0
FADRadd-8-3	pKFpurFKQPW	1030	0
FADRadd-8-3	pKFpurF	805	0

Example 3

1) Construction of desensitized type *purF* plasmid for homologous recombination

In order to introduce desensitized type *purF* substitution in a chromosome by using the *purF*⁻ strain produced in Example 1, Section 1), another *purF* fragment longer than the previously obtained *purF* fragment (about 1.6 kb) by about 0.5 kb for the 3' side was prepared. PCR was carried out (94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 and 29-mer primers for both ends, having nucleotide sequences of CTCCTGCAGAACGAGGAAAAAGACGTATG (SEQ ID NO: 1) and CTCAAGCTTGTCTGATTTATCACATCATC (SEQ ID NO: 13), and prepared based on the information of the gene data bank (*E. coli* Gene Bank), and an amplified fragment of about 2.1 kb including

the *purF* structural gene region covering SD-ATG and the translation termination codon was cloned into pCRTMII vector (Invitrogen). The plasmid contained in this clone is designated as pCRTMIIpurFL. The pCRTMIIpurFL has *Eco*RI sites as restriction sites at vicinities of the both sides of the cloning site. A *Pst*I site and a *Hind*III site are respectively provided in the PCR primers.

Then, the pCRTMIIpurFL was digested with *Sna*BI and *Hind*III to obtain a fragment of about 0.65 kb present downstream of the C-terminus of the *purF* coding region. This fragment was inserted between the *Sna*BI site and the *Hind*III site of pKFpurFKQ and pKFpurFKQPW obtained in Example 1, Section 5) to obtain pKFpurFLKQ and pKFpurFLKQPW.

Then, the pKFpurFLKQ and pKFpurFLKQPW were digested with *Eco*RI and *Hind*III to give fragments of about 2.1 kb containing *purFLKQ* and *purFLKQPW*. These fragments were inserted between the *Eco*RI and *Hind*III sites of pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (described above), to obtain plasmids pMAN997purFLKQ and pMAN997purFLKQPW, respectively.

2) Breeding of strain having desensitized type *purF* integrated in chromosome

The strain FDRadd-18-1 (*purF*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) and the strain FADRadd-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) were each transformed with the plasmids pMAN997purFLKQ and

pMAN997purFLKQPW at 30°C, and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin, and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. Among the ampicillin-sensitive clones, clones that were grown on the minimal medium were selected for the strain FDRadd-18-1 (*purF*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$), and clones that were grown in the minimal medium supplemented with 100 mg/L of L-histidine and 50 mg/L of adenine were selected for the

strain FADRadd-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$).

The fragments of about 1.5 kb including *purF* were amplified from chromosome DNA of these target clones, and nucleotide sequences around the locations where mutations were introduced by homologous recombination substitution were determined. As a result, it was confirmed that they contained the mutation of K326Q (326Lys→Gln), and the mutations of K326Q (326Lys→Gln) + P410W (410Pro→Trp), respectively.

Those derived from the strain FDRadd-18-1 (*purF*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) were designated as strain FDRadd-18-1::KQ (*purFKQ*, *deoD*$^-$, *purR*$^-$, *add*$^-$) and strain FDRadd-18-1::KQPW (*purFKQPW*, *deoD*$^-$, *purR*$^-$, *add*$^-$), and those derived from the strain FADRadd-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) were designated as strain FADRadd-8-3::KQ (*purFKQ*, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) and strain FADRadd-8-3::KQPW (*purFKQPW*, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$).

3) Evaluation of purine nucleoside-producing ability of strain having desensitized type *purF* integrated into chromosome

Purine nucleoside-producing abilities of the strain FDRadd-18-1::KQ (*purFKQ*, *deoD*$^-$, *purR*$^-$, *add*$^-$), the strain FDRadd-18-1::KQPW (*purFKQPW*, *deoD*$^-$, *purR*$^-$, *add*$^-$), the strain FADRadd-8-3::KQ (*purFKQ*, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) and the strain FADRadd-8-3::KQPW (*purFKQPW*, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) prepared in Section 2) were evaluated. The basal medium and

the culture method for the purine nucleoside production and the analysis method were the same as Example 1. For the strains of *purA*⁻ (adenine auxotrophic), 5 mg/L of adenine was added to the MS medium.

The results of the evaluation of purine nucleoside-producing ability are shown in Table 4. Superior inosine production was observed compared with the strain W3110 (wild type strain).

Table 4

Evaluation of purine nucleoside-producing ability

Strain	Purine nucleoside accumulation	
	Inosine (mg/L)	Guanosine (mg/L)
W3110	Trace	0
FDRadd-18-1::KQ	110	0
FDRadd-18-1::KQPW	105	0
FADRadd-8-3::KQ	635	0
FADRadd-8-3::KQPW	620	0

Example 4

1) Breeding of strain deficient in inosine-guanosine kinase gene (*gsk*)

PCR was carried out (94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 and 23-mer and 21-mer primers for both ends, having nucleotide sequences of CTCGAGCTCATGAAATTTCCCGG (SEQ ID NO: 14) and

CTCGGATCCGGTACCATGCTG (SEQ ID NO: 15), and prepared based on the information of a gene data bank (GenBank Accession No. D00798), and an amplified fragment of about 1.5 kb including the *gsk* structural gene region covering ATG and the translation termination codon was cloned between the *Sac*I site and the *Bam*HI site of pUC18 vector (Takara Shuzo). A *Sac*I site and a *Bam*HI site are respectively provided in the PCR primers.

The cloned *gsk* fragment of 1.5 kb contained one *Bgl*II site at about 830 bp from the 5' end, and therefore the plasmid was digested with *Bgl*II, and subjected to T4 DNA ligase reaction in order to insert a kanamycin resistant (Km^r) gene GenBlock (*Bam*HI digest, Pharmacia Biotech). Competent cells of *E. coli* JM109 were transformed with this ligation solution, and transformants grown on LB agar plates containing 50 μg/ml of kanamycin were obtained. Plasmid DNAs were prepared from the transformants of 4 clones, and a plasmid DNA that was not digested with *Bgl*II from which plasmid a fragment of about 2.8 kp was excised by *Eco*RI and *Sal*I digestion (pUCgsk'#2) was selected from the plasmid DNAs. The *gsk* contained in this plasmid has an inserted heterogeneous gene at the *Bgl*II site, and therefore it is predicted that the encoded enzyme lacks its function (Figure 2).

Then, the pUCgsk'#2 was digested with *Sac*I, *Sph*I and *Dra*I to prepare a fragment of about 2.8 Kb that included the *gsk* and Km^r genes. The *Dra*I digestion is employed to facilitate the preparation of a *Sac*I-*Sph*I fragment. The

fragment was inserted between the *Sac*I and *Sph*I sites of pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (describe above), to obtain plasmid pMAN997gsk'#2. The strain FDR-18 (*purF*$^-$, *deoD*$^-$, *purR*$^-$) and the strain FADRadd-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) were transformed at 30°C with the plasmid pMAN997gsk'#2, and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin, and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates, LB agar plates containing 25 μg/ml of ampicillin, respectively, and LB agar plates

containing 20 μg/ml of kanamycin, respectively, and clones not grown on the LB agar plates containing 25 μg/ml of ampicillin, but grown on the LB agar plates containing 20 μg/ml of kanamycin were selected. Furthermore, the fragment including the *gsk* gene was amplified by PCR from the chromosome DNA of these target clones, and it was confirmed that the about 2.8 kb fragment including Km^r gene, not the original fragment of about 1.5 kb, was amplified. It was also confirmed that the inosine-guanosine kinase activity was not detected in them. The inosine-guanosine kinase activity was measured according to the method of Usuda et al. (Biochim. Biophys. Acta., 1341, 200-206 (1997)). Those clones were considered strains deficient in *gsk*, and clones derived from the strain FDR-18 (*purF*$^-$, *deoD*$^-$, *purR*$^-$) and the strain FADRadd-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) are designated as strain FDRG-18-13 and strain FADRaddG-8-3, respectively.

2) Evaluation of purine nucleoside-producing ability of desensitized type *purF* plasmid-introduced strain

Because the plasmids pKFpurFKQ and pKFpurFKQPW have the Km^r gene as a drug selection marker and the host strains FDRG-18-13 (*purF*$^-$, *deoD*$^-$, *purR*$^-$, *gsk*$^-$) and FADRaddG-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *gsk*$^-$) prepared in Section 1) are made kanamycin resistant, it is difficult to obtain transformants by introducing pKFpurFKQ and pKFpurFKQPW into the strains FDRG-18-13 and FADRaddG-8-3 for the evaluation of the purine nucleoside-producing ability. Therefore, exchange of drug

selection marker genes of the plasmids pKFpurFKQ and pKFpurFKQPW was performed by using pUC18 vector having the ampicillin resistance gene (Takara Shuzo). Because the locational relationship between the lac promoter and the multi-cloning site is common to pKF18 and pUC18, *purFKQ* and *purFKQPW* fragments were excised from pKFpurFKQ and pKFpurFKQPW by using *Pst*I and *Hin*dIII, and these were inserted between the *Pst*I and *Hin*dIII sites of pUC18 to prepare pUCpurFKQ and pUCpurFKQPW. The hosts, the strains FDRG-18-13 and FADRaddG-8-3, were transformed with the pUCpurFKQ and pUCpurFKQPW, and the purine nucleoside-producing abilities of the recombinants were evaluated. The basal medium and the culture method for the purine nucleoside production and the analysis method were the same as Example 1. For the strains of *purA*$^-$ (adenine auxotrophic), 5 mg/L of adenine was added to the MS medium.

The results of the evaluation of the purine nucleoside-producing ability are shown in Table 5. From these results, it was revealed that the microorganisms accumulated guanosine as well as inosine when the deficiency of *gsk* was added.

Table 5

Evaluation of purine nucleoside-producing ability

Host	Plasmid	Purine nucleoside accumulation	
		Inosine (mg/L)	Guanosine (mg/L)
W3110	-	Trace	0
FDRG-18-13	pUCpurFKQ	105	139
FDRG-18-13	pUCpurFKQPW	108	93
FADRaddG-8-3	pUCpurFKQ	126	52
FADRaddG-8-3	pUCpurFKQPW	222	49

3) Breeding of strains having desensitized type *purF* integrated into chromosome and evaluation of purine nucleoside-producing ability

The strains FDRG-18-3 (*purF*$^-$, *deoD*$^-$, *purR*$^-$, *gsk*$^-$) and FADRaddG-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *gsk*$^-$) were transformed at 30°C with the plasmids pMAN997purFLKQ and pMAN997purFLKQPW, respectively. Some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin, and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then,

several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. From ampicillin-sensitive clones, clones grown on the minimal medium were further selected for the strain FDRG-18-13 (*purF*$^-$, *deoD*$^-$, *purR*$^-$, *gsk*$^-$), and clones grown in the minimal medium supplemented with 100 mg/L of L-histidine and 50 mg/L of adenine were selected for the strain FADRaddG-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *gsk*$^-$).

Chromosome DNAs of these target clones were prepared, and fragments of about 1.5 kb including *purF* were amplified by PCR, and sequenced around the locations where the mutations were introduced thorough substitution by homologous recombination. As a result, it was confirmed that they had a mutation of K326Q (326Lys→Gln) and K326Q (326Lys→Gln) + P410W (410Pro→Trp), respectively.

The strains derived from the strain FDRG-18-13 (*purF*$^-$, *deoD*$^-$, *purR*$^-$, *gsk*$^-$) were designated as strain FDRG-18-13::KQ (*purFKQ*, *deoD*$^-$, *purR*$^-$, *gsk*$^-$) and strain FDRG-18-13::KQPW

(*purFKQPW*, *deoD*$^-$, *purR*$^-$, *gsk*$^-$), and those derived from the strain FADRaddG-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *gsk*$^-$) were designated as strain FADRaddG-8-3::KQ (*purFKQ*, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *gsk*$^-$) and strain FADRaddG-8-3::KQPW (*purFKQPW*, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *gsk*$^-$).

The strain FADRaddG-8-3::KQ (*purFKQ*, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *gsk*$^-$) was given a private number AJ13334. This strain was deposited at National Institute of Bioscience and Human-Technology of Ministry of International Trade and Industry (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-0046 Japan) on June 24, 1997 as an international deposition under the Budapest treaty, and received an accession number FERM BP-5993.

The purine nucleoside-producing abilities of these four kinds of strains having desensitized type *purF* integrated into chromosome were evaluated. The basal medium and the culture method for the purine nucleoside production and the analysis method were the same as Example 1. For the strains of *purA*$^-$ (adenine auxotrophic), 5 mg/L of adenine was added to the MS medium.

The results of the evaluation of the purine nucleoside-producing ability are shown in Table 6. From these results, it was revealed that the microorganisms accumulated guanosine as well as inosine when the deficiency of *gsk* was added.

Table 6

Evaluation of purine nucleoside-producing ability

Strain	Purine nucleoside accumulation	
	Inosine (mg/L)	Guanosine (mg/L)
W3110	Trace	0
FDRG-18-13::KQ	150	140
FDRG-18-13::KQPW	145	125
FADRaddG-8-3::KQ	550	135
FADRaddG-8-3::KQPW	530	130

Example 5

1) Construction of wild type *purR* plasmid for homologous recombination and breeding of strain having reversed *purR*$^+$ integrated into chromosome

In Example 1, Section 4), the plasmid (pUCpurR) carrying the *purR* fragment of about 1.8 kb between the *Sal*I site and the *Sph*I site of pUC19 vector (Takara Shuzo) was obtained. The pUCpurR was digested with *Sac*I and *Sph*I to prepare a fragment of about 1.8 kb that included wild type *purR*. This fragment was inserted between the *Sac*I site and the *Sph*I site of pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (described above), to obtain plasmid pMAN997purR. The strain FADRadd-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) was transformed at 30°C with the plasmid pMAN997purR, and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin, and

cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. 10 clones were randomly selected from the ampicillin-sensitive clones, and the *purR* fragments of about 1.8 kb were amplified from the chromosome DNA of these clones by PCR. Clones in which the amplified fragment was digested with *Pma*CI but not with *Bgl*II were selected. The clones were considered *purR*$^+$ reversed strains, and designated as FADadd-8-3-2 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *add*$^-$).

2) Evaluation of purine nucleoside-producing ability of desensitized type *purF*-introduced strain

A transformant was produced by introducing pKFpurFKQ into the strain FADadd-8-3-2 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *add*$^-$), and purine nucleoside-producing ability of the strain was evaluated. For the strain FADRadd-8-3, a transformant with pKFpurKQ was also prepared, and an effect of purR deficiency was evaluated by comparison. The basal medium and the culture method for the purine nucleoside production and the analysis method were the same as Example 1. The MS medium was supplemented with 5 mg/L adenine.

The results of the evaluation of the purine nucleoside-producing ability are shown in Table 7. Superior inosine production was observed in FADRadd (*purR*$^-$ type) compared with FADadd (*purR* wild type) and the effect of *purR* deficiency was confirmed.

Table 7

Evaluation of purine nucleoside-producing ability

Host	Plasmid	Purine nucleoside accumulation	
		Inosine (mg/L)	Guanosine (mg/L)
W3110	-	Trace	0
FADRadd-8-3	pKFpurFKQ	1080	0
FADadd-8-3-2	pKFpurFKQ	930	0

Example 6

1) Rebreeding of strain deficient in inosine-guanosine kinase

gene (*gsk*)

PCR was carried out (94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 and 32-mer and 29-mer primers for both ends, having nucleotide sequences of CTCGGTACCCTGTTGCGTTAAGCCATCCCAGA (SEQ ID NO: 16) and CTCGCATGCCAACGTACGGCATTAACCTA (SEQ ID NO: 17), and prepared based on the information of a gene data bank (GenBank Accession No. D00798), and an amplified fragment of about 3.0 kb including the *gsk* structural gene region (about 800 bp) covering ATG and the translation termination codon was cloned between the *Kpn*I site and the *Sph*I site of pUC19 vector (Takara Shuzo). A *Kpn*I site and a *Sph*I site are respectively provided in the PCR primers.

The cloned *gsk* fragment of 3.0 kb contained two *Aro*51HI sites at about 900 bp and 1030 bp and one *Bgl*II site at about 1640 bp from the 5' end, and therefore the plasmid was digested with *Aro*51HI and *Bgl*II, and blunt-ended by T4 DNA polymerase. Then the *Aro*51HI-*Bgl*II fragment was removed and DNA of the vector was subjected to self-ligation by T4 DNA ligase. Competent cells of *E. coli* JM109 were transformed with this ligation solution, and transformants grown on LB agar plates containing 25 μg/ml of ampicillin were obtained. Plasmid DNAs were prepared from the transformants of 10 clones, and a plasmid DNA which was not digested with *Aro*51HI or *Bgl*II and from which plasmid a fragment of about 2.3 kp was excised by *Kpn*I and *Sph*I digestion (pUC19gsk'#10) was selected from

the plasmid DNAs. The *gsk* contained in this plasmid has a deletion in the structural gene between the *Aro*51HI site and the *Bgl*II site, and therefore it is predicted that the encoded enzyme lacks its function (Figure 3).

Then, the pUC19gsk'#10 was digested with *Kpn*I and *Sph*I to prepare a fragment of about 2.3 kb that included the *gsk* gene. The fragment was inserted between the *Kpn*I and *Sph*I sites of pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (describe above), to obtain plasmid pMAN997gsk'#10. The strain FADRadd-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) was transformed at 30°C with the plasmid pMAN997gsk'#10, and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin, and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture was appropriately diluted so that single colonies should be

obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. Furthermore, 10 clones were randomly selected from the ampicillin-sensitive clones, the fragments including the *gsk* gene were amplified by PCR using the above PCR primers from the chromosome DNA of these target clones, and the clones in which the fragment of about 2.3 kb, not the original fragment of about 3.0 kb were amplified were selected. It was also confirmed that the inosine-guanosine kinase activity was not detected in them. The inosine-guanosine kinase activity was measured according to the method of Usuda et al. (Biochim. Biophys. Acta., 1341, 200-206 (1997)). The clones were considered new strains deficient in *gsk*, and the clones derived from the strain FADRadd-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) were designated as FADRaddgsk (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *gsk*$^-$).

2) Breeding of strain deficient in GMP reductase gene (*guaC*)

PCR was carried out (94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 and 29-mer primers for both ends, having nucleotide sequences of CTCAAGCTTACGGCTCTGGTCCACGCCAG (SEQ ID NO: 18) and

CTCCTGCAGCAGCGTTGGGAGATTACAGG (SEQ ID NO: 19), and prepared based on the information of a gene data bank (*E. coli* Gene Bank), and an amplified fragment of about 2.2 kb including the *guaC* structural gene region covering SD-ATG and the translation termination codon was cloned between the *Hin*dIII site and the *Pst*I site of pUC18 vector (Takara Shuzo). A *Hin*dIII site and a *Pst*I site are respectively provided in the PCR primers.

The cloned *guaC* fragment of 2.2 kb contained one *Bgl*II site at about 1.1 kb from the 5' end, and therefore the plasmid was digested with *Bgl*II, blunt-ended by T4 DNA polymerase and ligated with T4 DNA ligase. Competent cells of *E. coli* JM109 were transformed with this ligation solution, and transformants grown on LB agar plates containing 25 μg/ml of ampicillin were obtained. Plasmid DNAs were prepared from the transformants of 18 clones, and a plasmid DNA from which a fragment of about 2.2 kp was excised by *Hin*dIII and *Pst*I digestion, and which fragment was not digested with *Bgl*II (pUC18guaC'#1) was selected from the plasmid DNAs. The *guaC* contained in this plasmid has a frame shift at the *Bgl*II site, and therefore it is predicted that the encoded enzyme lacks its function (Figure 3).

Then, the pUC18guaC'#1 was digested with *Hin*dIII and *Pst*I to prepare a fragment of about 2.2 kb that included *guaC*. The fragment was inserted between the *Hin*dIII and *Pst*I sites of pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori)

(describe above), to obtain plasmid pMAN997guaC'#1. The strain FADRadd-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) and the strain FADRaddgsk (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *gsk*$^-$) were transformed at 30°C with the plasmid pMAN997guaC'#1, and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin, and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. The fragments of about 2.2 kb including *guaC* were amplified by PCR from the chromosome DNA of these target

clones, and it was confirmed that the fragment was not digested with *Bgl*II. The clones satisfying the above conditions were considered strains deficient in *guaC*, and the clones derived from the strains FADRadd-8-3 and FADRaddgsk are designated as FADRaddguaC (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *guaC*$^-$) and FADRaddgskguaC (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *gsk*$^-$, *guaC*$^-$), respectively. It was also confirmed that the GMP reductase activity was not detected in them. The GMP reductase activity was measured according to the method of B. B. Garber et al. (J. Bacteriol., 43, 105(1980)).

3) Evaluation of purine nucleoside-producing ability of desensitized type *purF* plasmid-introduced strain

Transformants were produced by introducing pKFpurFKQ into the strain FADRaddguaC (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *guaC*$^-$) and FADRaddgskguaC (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *gsk*$^-$, *guaC*$^-$) produced in Section 2), and purine nucleoside-producing abilities of the strains were evaluated. The basal medium and the culture method for the purine nucleoside production and the analysis method were the same as Example 1. The MS medium was supplemented with 5 mg/L adenine.

The results of the evaluation of the purine nucleoside-producing ability are shown in Table 8. A certain level of improvement in guanosine production was observed by the deficiency of *guaC*.

Table 8

Evaluation of purine nucleoside-producing ability

Host	Plasmid	Purine nucleoside accumulation	
		Inosine (mg/L)	Guanosine (mg/L)
FADRadd-8-3	pKFpurFKQ	1080	0
FADRaddguaC	pKFpurFKQ	670	20
FADRaddgsk	pKFpurFKQ	920	140
FADRaddgskguaC	pKFpurFKQ	750	180

Example 7

1) Breeding of strain deficient in 6-phosphogluconate dehydrase gene (*edd*)

PCR was carried out (94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 and 29-mer primers for both ends, having nucleotide sequences of CTCGAATTCGGATATCTGGAAGAAGAGGG (SEQ ID NO: 20) and CTCAAGCTTGGAATAGTCCCTTCGGTAGC (SEQ ID NO: 21), and prepared based on the information obtained through searching of a gene data bank (*E. coli* Gene Bank) using "edd" as a key word, and an amplified fragment of about 3.0 kb including the *edd* structural gene region covering ATG and the translation termination codon, about 810 bp 5' upstream region of ATG and about 360 bp downstream region of the translation termination codon was cloned into pCRTMII vector (Invitrogen) as it was. The amplified fragment of the PCR product can be cloned into this vector as it is. The vector has *Eco*RI sites

as restriction sites at vicinities of the both sides of the cloning site. A *Bam*HI site and a *Hind*III site are respectively provided in the PCR primers. The cloned *edd* fragment of 3.0 kb contained two *Stu*I sites at about 660 bp and 1900 bp from the 5' end, and therefore the plasmid was digested with *Stu*I. Then the *Stu*I fragment of about 1.25 kb was removed and DNA of the vector was subjected to self-ligation by T4 DNA ligase. Competent cells of *E. coli* HB101 were transformed with this ligation solution, and transformants grown on LB agar plates containing 25 μg/ml of ampicillin were obtained. Plasmid DNAs were prepared from the transformants of 10 clones, and a plasmid DNA from which a fragment of about 1.25 kp was not excised by *Stu*I (pCRTMIIedd'#1) was selected from the plasmid DNAs. The *edd* contained in this plasmid has a deletion of a protein-coding region including a promoter region, and therefore it is predicted that the enzyme is not formed (Figure 3).

Then, the pCRTMIIedd'#1 was digested with *Eco*RI to prepare a fragment of about 1.75 kb that included a part of *edd* and a flanking region thereof. The fragment was inserted into the *Eco*RI site of pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (describe above), to obtain plasmid pMAN997edd'#1. The strain FADRadd-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) was transformed at 30°C with the plasmid pMAN997edd'#1, and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin,

and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. The *edd* regions were amplified by PCR using the above PCR primers from the chromosome DNA of these target clones, and the clones in which the size of the amplified fragment is about 1.75 kb of deletion type, not about 3.0 kb of wild type were selected. The clones were considered strains deficient in *edd* and designated as FADRaddedd (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$).

2) Evaluation of purine nucleoside-producing ability of desensitized type *purF* plasmid-introduced strain

A transformant was produced by introducing pKFpurFKQ into the strain FADRaddedd (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$) bred in Section 1), and purine nucleoside-producing ability of the strain was evaluated. The basal medium and the culture method for the purine nucleoside production and the analysis method were the same as Example 1. The MS medium was supplemented with 5 mg/L adenine. The 6-phosphogulconate dehydrase encoded by *edd* is an enzyme which is induced by gluconic acid and positioned at the first step in the Entner-Doudoroff pathway metabolizing gluconate to pyruvate. Because the gluconate was considered to flow only into the pentose phosphate pathway by deficiency of this enzyme, gluconic acid (48 g/L added) was used as a carbon source other than glucose to carry out the evaluation.

The results of the evaluation of the purine nucleoside-producing ability are shown in Table 9. Remarkable improvement in inosine production was observed by the deficiency of *edd*, when the gluconic acid was used as the carbon source. The effect was also observed when glucose was used as the carbon source.

Table 9

Evaluation of purine nucleoside-producing ability

Host	Plasmid	Carbon source	Purine nucleoside accumulation	
			Inosine (mg/L)	Guanosine (mg/L)
FADRadd-8-3	pKFpurFKQ	Glucose	1080	0
FADRaddedd	pKFpurFKQ	Glucose	1340	0
FADRadd-8-3	pKFpurFKQ	Gluconic acid	1050	0
FADRaddedd	pKFpurFKQ	Gluconic acid	2600	0

Example 8

1) Breeding of strain deficient in phosphoglucose isomerase gene (*pgi*)

PCR was carried out (94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 and 29-mer primers for both ends, having nucleotide sequences of CTCGTCGACTCCATTTTCAGCCTTGGCAC (SEQ ID NO: 22) and CTCGCATGCGTCGCATCAGGCATCGGTTG (SEQ ID NO: 23), and prepared based on the information obtained through searching of a gene data bank (*E. coli* Gene Bank) using "pgi" as a key word, and an amplified fragment of about 2.2 kb including the *pgi* structural gene region covering ATG and the translation termination codon was cloned between the *Sal*I site and the *Sph*I site of pUC18 vector (Takara Shuzo). A *Sal*I site and a *Sph*I site are respectively provided in the PCR primers. The cloned *pgi* fragment of 2.2 kb contained one *Bss*HII site and one *Mlu*I site at about 1170 bp and 1660 bp from the 5'

end, respectively, and therefore the plasmid was digested with *Bss*HII and *Mlu*I, and blunt-ended by T4 DNA polymerase. Then the fragment of about 500 bp between the *Bss*HII site and the *Mlu*I site was removed and DNA of the vector was subjected to self-ligation by T4 DNA ligase. Competent cells of *E. coli* JM109 were transformed with this ligation solution, and transformants grown on LB agar plates containing 25 μg/ml of ampicillin were obtained. Plasmid DNAs were prepared from the transformants of 10 clones, and a plasmid DNA from which a fragment of about 1.7 kp was excised by *Sal*I and *Sph*I digestion (pUC18pgi'#1) was selected from the plasmid DNAs. The *pgi* contained in this plasmid has a deletion between the *Bss*HII site and the *Mlu*I site, and therefore it is predicted that the encoded enzyme lacks its function (Figure 3).

Then, the pUC18pgi'#1 was digested with *Sal*I and *Sph*I to prepare a fragment of about 1.7 kb that included *pgi*. The fragment was inserted between the *Sal*I site and the *Sph*I site of pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (describe above), to obtain plasmid pMAN997pgi'#1. The strain FADRadd-8-3 (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$) and the strain FADRaddedd (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$) were transformed at 30°C with the plasmid pMAN997pgi'#1, and some of the obtained colonies were each streaked on LB agar plates containing 25 μg/ml of ampicillin, and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that

single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. The *pgi* regions were amplified by PCR using the above PCR primers from the chromosome DNA of these target clones, and the clones in which the size of the amplified fragment was about 1.7 kb of deletion type, not about 2.2 kb of wild type were selected. The clones were considered strains deficient in *pgi*, and clones derived from FADRadd-8-3 and FADRaddedd were designated as FADRaddpgi (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *pgi*$^-$) and FADRaddeddpgi (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *pgi*$^-$), respectively.

2) Evaluation of purine nucleoside-producing ability of desensitized type *purF* plasmid-introduced strain

Transformants were produced by introducing pKFpurFKQ into the strain FADRaddpgi (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *pgi*$^-$) and the strain FADRaddeddpgi (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *pgi*$^-$) bred in Section 1), and purine nucleoside-producing abilities of the strains were evaluated. The basal medium and the culture method for the purine nucleoside production and the analysis method were the same as Example 1 provided that the amount of yeast extract in the MS medium (basal medium) which was a medium used for evaluation of production was increased to 0.8%.

The results of the evaluation of the purine nucleoside-producing ability are shown in Table 10. By deficiency of *pgi*, growth remarkably lowered in the MS medium supplemented with 5 mg/L of adenine which was used in the above Examples. Therefore, the medium in which the amount of yeast extract was increased to 0.8% was used. In this medium, the *pgi*$^+$ parent strain showed increase of the growth rate, lowering of inosine production and by-production of hypoxanthine. On the contrary, remarkable improvement in inosine production was observed in the strain deficient in *pgi*.

Table 10

Evaluation of purine nucleoside-producing ability

Host	Plasmid	Purine nucleoside accumulation	
		Inosine (mg/L)	Hypoxanthine (mg/L)
FADRadd-8-3	pKFpurFKQ	450	260
FADRaddpgi	pKFpurFKQ	2770	100
FADRaddedd	pKFpurFKQ	780	210
FADRaddeddpgi	pKFpurFKQ	3080	120

Example 9

1) Breeding of strain deficient in adenine deaminase gene (*yicP*)

In a gene data bank (*E.coli* Gene Bank), *yicP* is registered as ORF (open reading frame, structural gene) which has a high homology with adenine deaminase from *Bacillus subtilis*. PCR was carried out (94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 and 29-mer primers for both ends, having nucleotide sequences of CTCCTGCAGCGACGTTTTCTTTTATGACA (SEQ ID NO: 24) and CTCAAGCTTCGTAACTGGTGACTTTTGCC (SEQ ID NO: 25), and prepared based on the information obtained through searching using "yicP" as a key word, to amplify a fragment of about 1.9 kb including the *yicP* structural gene region covering ATG and the translation termination codon, about 50 bp 5' upstream region of ATG and about 40 bp downstream region of the translation termination codon. A *Pst*I site and a *Hin*dIII site

are respectively provided in the PCR primers. The PCR product was digested with *Pst*I and *Hin*dIII, and cloned between the *Pst*I site and the *Hin*dIII site of pUC18 vector (Takara Shuzo). The cloned *yicP* fragment of 1.9 kb contained one *Hap*I site and one *Eco*RV site at about 540 bp and 590 bp from the 5' end, respectively, and therefore the plasmid was digested with *Hap*I and *Eco*RV. Then the *Hap*I-*Eco*RV fragment of 47 bp was removed and DNA of the vector was subjected to self-ligation by T4 DNA ligase. Competent cells of *E. coli* JM109 were transformed with this ligation solution, and transformants grown on LB agar plates containing 25 μg/ml of ampicillin were obtained. Plasmid DNAs were prepared from the transformants of 10 clones, and a plasmid DNA which was not digested with *Hap*I or *Eco*RV (pUC18yicP'#1) was selected from the plasmid DNAs. The *yicP* contained in this plasmid has a frame shift due to a deletion of 47 bp of *Hap*I-*Eco*RV sites, and therefore it is predicted that the encoded enzyme lacks its function (Figure 3).

Then, the pUC18yicP'#1 was digested with *Pst*I and *Hin*dIII to prepare a fragment of about 1.9 kb that included the *yicP* gene. The fragment was inserted between the *Pst*I site and the *Hin*dIII site of pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (describe above), to obtain plasmid pMAN997yicP'#1. The strain FADRaddedd (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$) was transformed at 30°C with the plasmid pMAN997yicP'#1, and some of the obtained colonies

were streaked on LB agar plates containing 25 μg/ml of ampicillin, and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. The *yicP* regions were amplified by PCR using the above PCR primers from the chromosome DNA of these target clones, and the clones in which the size of the amplified fragment was not digested with *Hap*I or *Eco*RV were selected. It was also confirmed that the adenine deaminase activity was not detected in these clones. The adenine deaminase

activity was measured according to the method of Per Nygaard et al. (J. Bacteriol., 178, 846-853 (1996)). The clones were considered strains deficient in *yicP*, and designated as FADRaddeddyicP (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *yicP*$^-$).

2) Breeding of strain deficient in phosphoglucose isomerase gene (*pgi*) from strain deficient in adenine deaminase gene (*yicP*)

The deficiency of *pgi* was also added to the strain FADRaddeddyicP (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *yicP*$^-$). By using pMAN997pgi'#1 constructed in Example 8, a strain FADRaddeddyicPpgi (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *yicP*$^-$, *pgi*$^-$) was obtained in the same method as in Example 8.

3) Evaluation of purine nucleoside-producing ability of desensitized type *purF* plasmid-introduced strain

Transformants were produced by introducing pKFpurFKQ into the strain FADRaddeddyicP (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *yicP*$^-$) and the strain FADRaddeddyicPpgi (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *yicP*$^-$, *pgi*$^-$) bred in Sections 1) and 2), and responses of growth to an adenine amount and purine nucleoside-producing abilities of the strains were evaluated. The basal medium and the culture method for the purine nucleoside production and the analysis method were the same as Example 1 provided that the used medium was the MS medium to which adenine was added in an amount between 0 to 50 mg/L.

The results of the evaluation of the growth response to adenine and the purine nucleoside-producing ability are shown in Table 11. By deficiency of *yicP*, the growth rate with respect to adenine was improved and an effect of the deficiency of *yicP* was observed when adenine was added in amounts of 50 mg/L and 20 mg/L.

Table 11

Evaluation of purine nucleoside-producing ability

Host	Plasmid	Adenine added (mg/L)	Growth rate (OD)	Purine nucleoside accumulation	
				Inosine (mg/L)	Hypoxanthine (mg/L)
FADRaddedd	pKFpurFKQ	0	2.2	870	0
		50	3.2	650	0
FADRaddeddyicP	pKFpurFKQ	0	2.4	870	0
		50	6.8	1100	40
FADRaddeddpgi	pKFpurFKQ	5	2.2	1420	28
		20	3.4	1760	48
FADRaddeddyicPpgi	pKFpurFKQ	5	2.1	1380	7
		20	3.7	2350	19

Example 10

1) Preparation of PRPP synthetase gene (*prs*)

PCR was carried out (94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 and 38-mer and 29-mer primers for both ends, having nucleotide sequences of CTCGTCGACTGCCTAAGGATCTTCTCATGCCTGATATG (SEQ ID NO: 26) and CTCGCATGCGCCGGGTTCGATTAGTGTTC (SEQ ID NO: 27), and prepared based on the information of a gene data bank (*E.coli* Gene Bank), and an amplified fragment of about 1 kb including

the *prs* structural gene region covering SD-ATG and the translation termination codon was cloned into pUC18 vector (Takara Shuzo). A *Sal*I site and a *Sph*I site are respectively provided in the PCR primers. The PCR product was digested with *Sal*I and *Sph*I, and cloned between the *Sal*I site and the *Sph*I site of pUC18 vector (pUCprs).

2) Construction of desensitized type *prs*

A *prs* fragment was excised from the plasmid carrying the *prs* of about 1 kb cloned in Section 1) by *Sal*I and *Sph*I digestion, and inserted between the *Sal*I and *Sph*I sites of the multi-cloning site of a plasmid for introducing mutation, pKF19k (Takara Shuzo) to obtain the target clone (pKFprs). S. G. Bower et al. (J. Biol. Chem., 264, 10287 (1989)) has suggested that PRPP synthetase (Prs) is subjected to feedback inhibition by AMP and ADP. It is also described that the enzyme whose Asp (D) at position 128 is mutated to Ala (A) is partially desensitized. Therefore, the following synthetic DNA primer was prepared for gene substitution realizing mutation of Asp (D) at position 128 PRPP synthetase (Prs) to Ala (A), and pKFprs was subjected to site-directed mutagenesis according to the protocol of Site-directed Mutagenesis System Mutan-Super Express Km (Takara Shuzo) to introduce a site-directed mutation into the pKFprs.

Primer for D128A mutation:

5'-GCGTGCAGAGCCACTATCAGC-3' (SEQ ID NO: 28)

After the mutagenesis, 12 clones were randomly picked

up from the resulting transformants, and plasmids were produced from them. By nucleotide sequencing of the plasmids around the locations where the mutations were introduced, it was confirmed that 9 clones of target mutants were obtained. The *prs* fragment was excised with *Sal*I and *Sph*I from pKFprsDA having the mutant type *prs*, and inserted between the *Sal*I site and the *Sph*I site of pUC18 and pSTV18 (Takara Shuzo). For using the wild type *prs* as a control, the *prs* fragment was excised with *Sal*I and *Sph*I from pUCprs constructed in the above, and inserted between the *Sal*I site and the *Sph*I site of pSTV18 (Takara Shuzo). Each of the plasmids pUCprsDA and pSTVprsDA and the plasmids pUCprs and pSTVprs has an inserted mutant *prs* or wild type *prs* downstream of the lacp/o (promoter of lactose operon) derived from pUC18 and pSTV18, respectively, and the *prs* is expressed under the control of this promoter.

Recombinant bacteria obtained by transforming *E. coli* JM109 cells with the above four plasmids were cultured in LB liquid medium for eight hours, and collected, and crude enzyme extracts were prepared from them. The PRPP synthetase activity of the extracts and degrees of inhibition by ADP were measured according to the method of K. F. Jensen et al. (Analytical Biochemistry, 98, 254-263 (1979)) which was partially modified. Specifically, [a-^{32}P]ATP was used as the substrate and [^{32}P]AMP produced by the reaction was measured. The results are shown in Table 12.

Table 12

PRPP synthetase (Prs) activity

Host	Plasmid	Property	Specific activity (nmole/min/mg crude enzyme extract)	
			None	5 mM ADP
JM109	pUC18	Control	2.9	ND
JM109	pUCprs	High copy, wild type	75.9	ND
JM109	pUCprsDA	High copy, mutant type	80.8	20.2
JM109	pSTVprs	Medium copy, wild type	11.5	ND
JM109	pSTVprsDA	Medium copy, mutant type	10.6	2.7

3) Evaluation of purine nucleoside-producing ability of desensitized type *prs* plasmid-introduced strain

Strains each having two plasmids simultaneously were made by introducing pKFpurFKQ into the strain FADRaddeddyicPpgi (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *yicP*$^-$, *pgi*$^-$) bred in Example 9, Section 3) to obtain a transformant and further each introducing pSTVprs and pSTVprsDA carrying *prs* and *prsDA* genes into the transformant, and purine nucleoside-producing abilities of the strains were evaluated. The basal medium and the culture method for the purine nucleoside production and the analysis method were the same as Example 1 provided that the amount of yeast extract in the MS medium was increased to 0.4%.

The results of the evaluation of the purine nucleoside-producing ability are shown in Table 13. By introduction of

mutant *prsDA* as a plasmid, an effect of improvement in inosine production was observed.

Table 13

Evaluation of purine nucleoside-producing ability

Host	Plasmid	Purine nucleoside accumulation	
		Inosine (mg/L)	Hypoxanthine (mg/L)
FADRaddeddyicPpgi	pKFpurFKQ	1600	8
	pKFpurFKQ+ pSTVprs	1450	3
	pKFpurFKQ+ pSTVprsDA	1815	10

Example 11

1) Breeding of strain deficient in xanthosine phosphorylase gene (*xapA*)

A gene inactivated by mutation was constructed in one step by Cross-over PCR using four primers prepared based on the information obtained through searching of a gene data bank (*E. coli* Gene Bank) using "xapA" as a key word. The used primers are as follows:

N-out: 5'-CGCGGATCCGCGACATAGCCGTTGTCGCC-3' (SEQ ID NO: 29)

N-in: 5'-CCCATCCACTAAACTTAAACATCGTGGCGTGAAATCAGG-3' (SEQ ID NO: 30)

C-in: 5'-TGTTTAAGTTTAGTGGATGGGCATCAACCTTATTTGTGG-3' (SEQ ID NO: 31)

C-out: 5'-CGCAAGCTTCAAACTCCGGGTTACGGGCG-3' (SEQ ID NO: 32)

First, PCR was carried out (94°C, 30 sec; 55°C, 1 min;

72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 and primers N-out (29-mer) and N-in (39-mer) as well as primers C-in (39-mer) and C-out (29-mer) for both ends, to obtain two PCR products (both are fragments of about 850 bp), respectively. Then, PCR was again carried out by using a mixture of the two PCR products as a template and the primers N-out and C-out for both ends, to amplify a gene fragment in which the gene region including the *xapA* structural gene region was shortened from a fragment of about 2.4 kb (size of wild type) to a fragment of about 1.7 kb. A *Bam*HI site and a *Hin*dIII site are provided in the PCR primers N-out and C-out, respectively. This PCR product was digested with *Bam*HI and *Hin*dIII, and the obtained fragment was ligated by T4 DNA ligase with a plasmid obtained by digesting pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (describe above), with *Bam*HI and *Hin*dIII. Competent cells of *E. coli* JM109 were transformed with this ligation solution, and transformants grown on LB agar plates containing 25 μg/ml of ampicillin were obtained. Plasmid DNAs were prepared from the transformants of 10 clones, and a plasmid DNA from which a fragment of about 1.7 kp was excised by *Bam*HI and *Hin*dIII digestion (pMAN997xapA'#1) was selected from the plasmid DNAs. The *xapA* contained in this plasmid has a deletion of about 700 bp in the structural gene, and therefore it is predicted that the encoded enzyme lacks its function (Figure 3).

The strain FADRaddeddyicPpgi (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *yicP*$^-$, *pgi*$^-$) was transformed at 30°C with the plasmid pMAN997xapA'#1, and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin, and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. The *xapA* region were amplified by PCR using the above PCR primers N-out and C-out from the chromosome DNA of these target clones, and the clones in which the size of the amplified fragment was about 1.7 kb were

selected. The clones were considered strains deficient in *xapA*, and designated as FADRaddeddyicPpgixapA (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *yicP*$^-$, *pgi*$^-$, *xapA*$^-$). In the strain deficient in *xapA*, xanthine production in a medium was not observed by culture with xanthosine supplemented, and it was confirmed that xanthosine phosphorylase was not induced. The xanthosine phosphorylase activity was measured according to the method of K. Hammer Jespersen et al. (Molec. Gen. Genet., 179, 341-348 (1980)).

2) Evaluation of purine nucleoside-producing ability of desensitized type *purF* plasmid-introduced strain

A transformant was produced by introducing pKFpurFKQ into the strain FADRaddeddyicPpgixapA (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *yicP*$^-$, *pgi*$^-$, *xapA*$^-$) bred in Section 1), and purine nucleoside-producing ability of the strain was evaluated. The basal medium and the culture method for the purine nucleoside production and the analysis method were the same as Example 1 provided that the MS medium in which the amount of yeast extract was increased to 0.8% was used.

The results of the evaluation of the purine nucleoside-producing ability are shown in Table 14. When the amount of yeast extract in the MS medium was increased, by-production of hypoxanthine which remarkably occurred after sugar consumption in the latter half of the culture was reduced by deficiency of *xapA*, and improvement of inosine production was observed.

Table 14

Evaluation of purine nucleoside-producing ability

Host	Plasmid	Culture period (days)	Purine nucleoside accumulation: Inosine (mg/L)	Purine nucleoside accumulation: Hypoxanthine (mg/L)
FADRaddeddyicPpgi	pKFpurFKQ	3	4640	146
		6	1850	1500
FADRaddeddyicPpgixapA	pKFpurFKQ	3	5870	57
		6	3810	915

Example 12

1) Breeding of strain deficient in nucleoside permease gene (*nupG*)

PCR was carried out(94°C, 30 sec; 55°C, 1 min; 72°C, 2 min; 30 cycles; Gene Amp PCR System Model 9600, Perkin Elmer) by using the chromosome DNA of the strain W3110 as a template and 35-mer primers for both ends, having nucleotide sequences of CTCGAATTCATGGTGCCGAACCACCTTGATAAACG (SEQ ID NO: 33) and CTCGTCGACATGCCGAAACCGGCGAATATAGCGAC (SEQ ID NO: 34), and prepared based on the information of a gene data bank (*E. coli* Gene Bank), to amplify a fragment of about 2.7 kb of an *nupG* structural gene region covering SD-ATG and the translation termination codon. An *Eco*RI site and a *Sal*I site are respectively provided in the PCR primers. The amplified fragment was digested with *Eco*RI, *Sal*I and *Afl*III. Since the PCR-amplified fragment contained two AflII sites, three fragments of about 750 bp, 820 bp and 1130 bp were formed.

The two fragments of about 720 bp and 1130 bp other than the *Afl*III fragment of about 820 bp were collected and were ligated by T4 DNA ligase with DNA obtained by digesting pUC18 vector (Takara Shuzo) with *Eco*RI and *Sal*I. Competent cells of *E. coli* HB101 were transformed with this ligation solution, and plasmid DNAs were prepared from 16 of the emerged colonies, and a plasmid DNA in which a fragment digested with *Eco*RI and *Sal*I was of about 1.9 kb (pUC18nupG'#1) was selected from the plasmid DNAs. The pUC18nupG'#1 was digested with *Eco*RI and *Sal*I, and the resulting fragment of about 1.9 kb was ligated by T4 DNA ligase with a plasmid obtained by digesting pMAN997, which is a vector for homologous recombination having a temperature-sensitive replication origin (tsori) (described above), with *Eco*RI and *Sal*I. Competent cells of *E. coli* JM109 were transformed with this ligation solution, and transformants grown on LB agar plates containing 25 μg/ml of ampicillin were obtained. Plasmid DNAs were prepared from the transformants of 10 clones, and a plasmid DNA from which a fragment of about 1.9 kp was excised by *Eco*RI and *Sal*I digestion (pMAN997nupG'#1) was selected from the plasmid DNAs. The *nupG* contained in this plasmid DNA has a deletion of about 820 bp in the structural gene, and therefore it is predicted that the encoded enzyme lacks its function (Figure 3).

The strain FADRaddeddyicPpgi (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *yicP*$^-$, *pgi*$^-$) was transformed at 30°C with the plasmid pMAN997nupG'#1, and some of the obtained colonies were streaked on LB agar plates containing 25 μg/ml of ampicillin,

and cultured at 30°C overnight. Then, the cultured bacterial cells were plated on LB agar plates containing 25 μg/ml of ampicillin so that single colonies should be obtained, to obtain colonies grown at 42°C. The procedure for obtaining single colonies grown at 42°C was repeated once, and clones in which the whole plasmid was integrated into the chromosome through homologous recombination were selected. It was confirmed that these clones did not have the plasmid in their cytoplasm. Then, several clones among these clones were streaked on LB agar plates, cultured at 30°C overnight, then inoculated into LB liquid medium (3 ml/test tube), and cultured at 42°C for 3 to 4 hours with shaking. The culture was appropriately diluted so that single colonies should be obtained (about 10^{-5} to 10^{-6} dilution), plated on LB agar plates, and cultured at 42°C overnight to obtain colonies. One hundred colonies were randomly picked up from the emerged colonies, and each allowed to grow on LB agar plates and LB agar plates containing 25 μg/ml of ampicillin, respectively, and ampicillin-sensitive clones grown only on the LB agar plates were selected. The *nupG* region were amplified by PCR using the above PCR primers from the chromosome DNA of these target clones, and clones in which the size of the amplified fragment was about 1.9 kb were selected. These clones were considered strains deficient in *nupG*, and designated as FADRaddeddyicPpginupG (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *yicP*$^-$, *pgi*$^-$, *nupG*$^-$).

2) Evaluation of purine nucleoside-producing ability of desensitized type *purF*-introduced strain

A transformant was made by introducing pKFpurFKQ into the strain FADRaddeddyicPpginupG (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *yicP*$^-$, *pgi*$^-$, *nupG*$^-$) bred in Section 1), and purine nucleoside-producing ability of the strain was evaluated. The basal medium and the culture method for the purine nucleoside production and the analysis method were the same as Example 1 provided that the MS medium in which the amount of yeast extract was increased to 1.2%.

The results of the evaluation of the purine nucleoside-producing ability are shown in Table 15. When the amount of yeast extract in the MS medium was increased, by-production of hypoxanthine which remarkably occurred after sugar consumption in the latter half of the culture was reduced and improvement of inosine production was observed by deficiency of *nupG*.

Table 15

Evaluation of purine nucleoside-producing ability

Host	Plasmid	Purine nucleoside accumulation	
		Inosine (mg/L)	Hypoxanthine (mg/L)
FADRaddeddyicPpgi	pKFpurFKQ	1190	835
FADRaddeddyicPpginupG	pKFpurFKQ	3390	315

Industrial Applicability

According to the present invention, a purine nucleoside-producing bacterium is created by derepressing and desensitizing an enzyme which subjected to the control in purine nucleoside biosynthesis and further blocking a decomposition system and a conversion system. The created purine nucleoside-producing bacterium can be suitably used for production of a purine nucleoside by fermentation.

What is claimed is:

1. A microorganism belonging to the genus *Escherichia* and having purine nucleoside-producing ability.

2. The microorganism according to claim 1, which has acquired the purine nucleoside-producing ability because of an increase of an activity of an enzyme involved in purine nucleoside biosynthesis in cells of the microorganism.

3. The microorganism according to claim 1, which has acquired the purine nucleoside-producing ability because of an increase of an expression amount of a gene for an enzyme involved in purine nucleoside biosynthesis.

4. The microorganism according to claim 1, which has acquired the purine nucleoside-producing ability because of deregulation of control of an enzyme involved in purine nucleoside biosynthesis.

5. The microorganism according to claim 4, the control of the enzyme involved in the purine nucleoside biosynthesis is desensitized by desensitization of feedback inhibition.

6. The microorganism according to any one of claims 3-5, wherein the enzyme involved in the purine nucleoside biosynthesis is phosphoribosyl pyrophosphate amidotransferase.

7. The microorganism according to claims 3 or 4, wherein the enzyme involved in the purine nucleoside biosynthesis is phosphoribosyl pyrophosphate synthetase.

8. The microorganism according to claim 4, wherein the control of the enzyme involved in the purine nucleoside biosynthesis is derepressed by inactivation of a purine

repressor.

9. The microorganism according to claim 1, which has acquired the purine nucleoside-producing ability because of blockage of a reaction branching from purine nucleoside biosynthesis and leading to another metabolite.

10. The microorganism according to claim 9, wherein the reaction branching from the purine nucleoside biosynthesis and leading to another metabolite is a reaction catalyzed by an enzyme selected from the group consisting of succinyl-adenosine monophosphate synthase, purine nucleoside phosphorylase, adenosine deaminase, inosine-guanosine kinase, guanosine monophosphate reductase, 6-phosphogluconate dehydrase, phosphoglucose isomerase, adenine deaminase, and xanthosine phosphorylase.

11. The microorganism according to claim 1, which is enhanced in the purine nucleoside-producing ability by weakening of incorporation of a purine nucleoside into cells of the microorganism.

12. The microorganism according to claim 11, wherein the incorporation of the purine nucleoside into cells of the microorganism is weakened by blockage of a reaction involved in the incorporation of the purine nucleoside into cells of the microorganism, and the reaction involved in the incorporation of the purine nucleoside into cells of the microorganism is a reaction catalyzed by nucleoside permease.

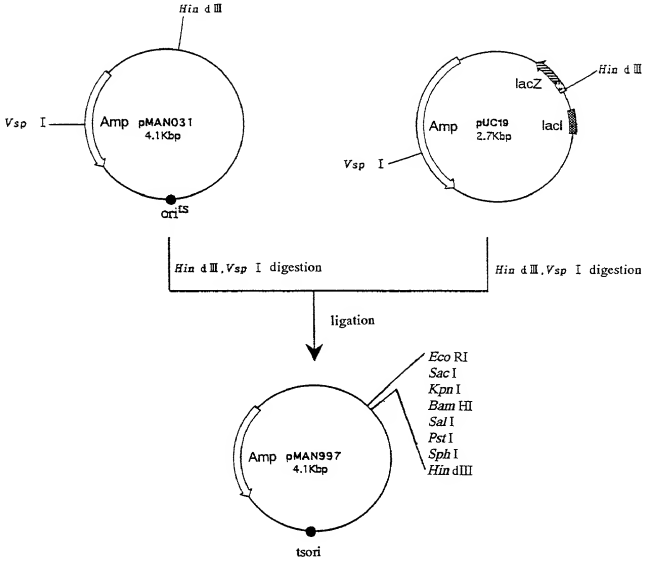
13. A method for producing a purine nucleoside by fermentation comprising culturing the microorganism as

defined in any one of claims 1-12 in a culture medium to produce and accumulate the purine nucleoside in the medium, and collecting the purine nucleoside.

Abstract of the Disclosure

A microorganism which has a gene encoding an enzyme in which feedback inhibition is desensitized by substitution of one or two amino acids in PRPP amidotransferase encoded by *purF* of *Escherichia coli*, a gene encoding a protein which is an inactivated repressor of purine nucleotide biosynthesis encoded by *purR*, a gene encoding an enzyme which is inactivated purine nucleoside phosphorylase encoded by *deoD*, a gene encoding an enzyme which is inactivated succinyl-AMP synthase encoded by *purA*, a gene encoding an enzyme which is inactivated 6-phosphogluconate dehydrase encoded by *edd*, a gene encoding an enzyme which is inactivated phosphoglucose isomerase encoded by *pgi* and like is bred and a purine nucleoside is produced by culturing the microorganism.

1 / 3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Hin d III, Vsp I digestion
ligation
Eco RI
Sac I
Kpn I
Bam HI
Sal I
Pst I
Sph I
Hin dIII
Amp pMAN997
4.1Kbp
tsori

FIG. 1

purF
Bgl II (fill in)
880
1530
purA
Hpa I
520
deletion
Sna B I
710
1300
deoD
Hpa I (Cla I linker)
680
1350
purR
Pma C I (Bgl II linker)
810
1770
add
Stu I (Bgl II linker)
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Bgl II (Bam HI)
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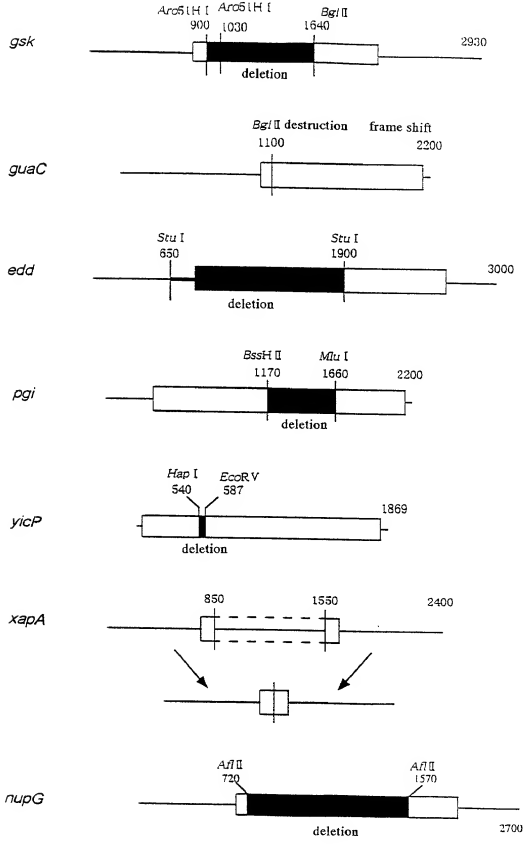
FIG. 2

3/3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deletion
pgi
BssH II
1170
Mlu I
1660
2200
deletion
yicP
Hap I
540
EcoR V
587
1869
deletion
xapA
850
1550
2400
nupG
Afl II
720
Afl II
1570
deletion
2700

FIG. 3

Declaration, Power Of Attorney and Petition

WE (I), the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD FOR PRODUCING PURINE NUCLEOSIDE BY FERMENTATION

the specification of which

☒ is attached hereto.

☐ was filed on _____ as

Application Serial No. _____

and amended on _____.

☐ was filed as PCT international application

Number PCT/JP 98/03239

on July 17, 1998,

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
9-194603	Japan	18/07/1997	☒ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application Number) (Filing Date)

(Application Number) (Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (pending, patented, abandoned)

And we (I) hereby appoint: Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Robert F. Gnuse, Registration Number 27,295; Jean-Paul Lavalleye, Registration Number 31,451; Timothy R. Schwartz, Registration Number 32,171; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,893; Richard L. Treanor, Registration Number 36,379; Steven P. Weihrouch, Registration Number 32,829; John T. Goolkasian, Registration Number 26,142; Marc R. Labgold, Registration Number 34,651; William J. Healey, Registration Number 36,160; and Richard L. Chinn, Registration Number 34,305; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1-00

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Sequence Listings

<110> AJINOMOTO Co., Inc.

<120> METHOD FOR PRODUCING PURINE NUCLEOSIDE BY FERMENTATION

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